BIOPROCESSING OF MICROALGAE FOR BIOENERGY AND RECOMBINANT PROTEIN PRODUCTION

A Dissertation

by

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ABSTRACT

This dissertation investigates harvesting of marine microalgae for bioenergy and production of two recombinant proteins for therapeutic applications in *Chlamydomonas reinhardtii.* The first study describes harvesting of marine microalgae by flocculation using aluminum chloride ($AlCl_3$), natural polymer chitosan, and synthetic cationic polymers.

Harvesting and concentration process of low concentration microalgae cultures ranging from 1 to 2 g dry weight per liter was affected by algogenic organic matter (AOM), ionic strength, cell concentration, polymer charge density, and media pH. Marine microalgae flocculation was greatly affected by the presence of AOM independently of the flocculant chemistry. Presence of AOM demanded extra flocculant dosage i.e., 3-fold of $AlCl_3$, 7-fold of highly charged synthetic cationic polymer, and 10-fold of chitosan. Flocculant dosage required for > 90 % flocculation efficiency in the presence of AOM was 160 mg/L, 50 mg/L, and 20 mg/L when using $AlCl_3$, chitosan, and best (more efficient) synthetic polymer respectively. The high-ionic strength of saline water did not have a significant effect on flocculation efficiency when using $AlCl_3$. However, to achieve efficient algal biomass removal, application of highly-charged synthetic polymers was required to overcome the presence of electrolytes. The best synthetic cationic polymer tested herein, which achieved greater than 90 % flocculation efficiency at 20 mg/L dosage, was a polymer with 99 % cationic charge density. Cell concentration also affected flocculant dosage requirement; low density

cultures (10^6 cells/mL) required 6-fold greater dosages than cultures grown until early stationary phase (10^7 cells/mL).

The second study addresses cultivation, extraction and purification challenges of two complex recombinant proteins, an immunotoxin molecule (MT51) and malaria vaccine antigen (Pfs25) produced in the chloroplast of *C. reinhardtii*. Main challenges identified were i) low transgene expression level, ii) proteolytic instability of MT51 immunotoxin, and iii) aggregation of Pfs25 antigen. Optimal expression and accumulation of Pfs25 antigen required growing *C. reinhardtii* cultures to late exponential phase (10^6 cells/mL) and inducing transgene expression for 24 h at a photon irradiance of 120 μmol/m^2s.

DEDICATION

To my beloved family for their unconditional love and support during all these years

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NOMENCLATURE

ADC	Antobody-drug conjugates
AFDW	Ash free dry weight
A. formosa	*Asterionella Formosa*
ALCL	Anaplastic large cell lymphoma
$AlCl_3$	Aluminum chloride
$Al(OH)_3$	Aluminum hydroxyde
$Al_2(SO_4)_3$	Aluminum sulphate
AOM	Algogenic organic matter
AP	Alkaline phosphatase
Ca	Calcium
$Ca(OH)_2$	Calcium hydroxide
CF	Concentration factor
CV	Column volume
C. vulgaris	*Chlorella vulgaris*
DT	Diptheria toxin
EOM	Extracellular organic matter
ETA	Exotoxin A
FDA	Food and Drug Administration
$FeCl_3$	Ferrum chloride
HCH23	Constant domain 2 and 3

HCl	Hydrochloric acid
HL	Hodking's lymphoma
IL2	Interleuking 2
Kan	Kanamycin
KH_2PO_4	Potasium phosphate
KOH	Potasium hydroxide
M. aeruginosa	*Microcystis aeruginosa*
Mg	Magnesium
MMAE	Microtubule disrupting agent monomethylauristatin E
MW	Molecular weight
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium azaide
$NaNO_3$	Sodium nitrate
Na_2SiO_3*9 H_2O	Sodium metasilicate nonahydrate
NH_4OH	Amonium hydroxide
N. oculata,	*Nannochlorisoculata*
NOM	Natural organic matter
N. salina	*Nannochloropsissalina*
OD_f	Optical density after 1 h settling
OD_o	Initial optical density at time zero
PBR	Photobioreactors

PBS	Phosphate-buffered saline
Pfs25	*Plasmodium falciparum* surface protein 25
Pfs28	*Plasmodium falciparum* surface protein 28
PPF	Photosynthetic photon flux
RE	Removal efficiency
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
scFv	Single chain antibody
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAP	Tris-acetate-phosphate
TBS	Tris-buffered saline
TSP	Total soluble protein
V_0	Initial volume (mL)
V_f	Final volume (mL)

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Bioprocessing cost and scalability of identified unit operations using microalgae as production systems of recombinant proteins and high-value native algae products (lutein, carotenoids, pigments, DHA and EPA oils, phytosterols, etc.) are uncertain. Possible explanations to this matter are: low yield production strains, poorly understood and controlled environmental factors triggering product synthesis, unoptimized bioreactor and biomass recovery, and in the case with recombinant proteins, early stage of platform development. The operational constrains on bioprocessing of microalgae for low-cost and large volumes of triglycerides and high–value therapeutics protein products are very different and require individual considerations.

Green microalgae can generate substantial quantities of triglycerides (as much as 40-60 % of dry cell weight) and thus provide an attractive alternative for producing oil for commodity applications in a renewable manner. However, biofuels production from green microalgae is still not economically feasible because of nutrients volume demand and cost, as well as costly algae harvesting and dewatering. According to Uduman et al. (2010) almost 40 % of the total microalgae manufacturing cost are related to downstream processes such as, harvesting algal biomass, biomass concentration and dewatering, and product extraction. Harvesting of microalgae slurries is the first step of dewatering process before oil extraction or thermochemical conversion of concentrated

wet biomass, accounting for at least half of the above cited 40 % downstream processing cost. Several physical, chemical, and mechanical methods have been tested for harvesting microalgae including membrane filtration, centrifugation, reverse-flow vacuum filtration, air flotation, acoustics-driven flocculation, and polyelectrolyte flocculation (Chen et al., 2009b). Flocculation using inorganic and polymer electrolytes is widely applied in water treatment, and has been successfully applied for dewatering of fresh water microalgae. However, the same technology does not seem to work that well for marine microalgae as past reports indicate reduction of flocculation efficiency due to high salt (ionic strength) in culture media. The latter effect is one of objectives in this dissertation i.e., understanding the effect of ionic strength and polyelectrolytes on flocculation efficiency of marine microalgae.

Several recombinant proteins with potential therapeutic applications have been recently (successfully) expressed in *C. reinhardtii* chloroplast at concentrations that permit an initial assessment of process factors and constraints posed by both, algal cultivation (upstream processing) and primary recovery (downstream processing). In this dissertation, bioprocessing challenges of two recombinant therapeutic proteins i) immunotoxin (antibody-drug conjugate) and ii) subunit malaria vaccine antigen, are being evaluated. The expression cassette designed by our collaborator to replace endogenous *psbA* gene with the transgenes regulated by endogenous *psbA* promoter provides unique opportunity for decoupling biomass production and protein accumulation. Hence, cultivation conditions for optimal *C. reinhardtii* biomass

accumulation and induced recombinant protein expression are reported and discussed in Chapter IV of the dissertation.

This dissertation addresses two different industrial bioprocess technology challenges and applications: 1) recovery of algal biomass for biofuels by flocculation and sedimentation (Chapters II and III) and 2) production and recovery of *C. reinhardtii*-expressed recombinant therapeutic proteins (Chapter IV). These two bioprocessing projects are closely related from a downstream processing viewpoint as both involve product separation and concentration unit operations. The process for algal biomass harvesting and concentration is cost driven and requires application of a low-cost and high-efficiency separation method. The bioprocessing of therapeutic proteins is not currently cost constrained, but faced with the novelty and complexity of the cell lysate. The overall goal of this dissertation was to understand and identify potential barriers to develop downstream processes using green microalgae as a platform for biofuel productions and high-value recombinant proteins.

1.2 Literature review

1.2.1 Fundamentals of coagulation and flocculation phenomena

Coagulation term is typically used for chemical destabilization of colloids due to a reduction in the zeta potential by changing the concentration of the ions in suspension (Rushton et al., 2000). Coagulation phenomena depends on the balance between the attraction forces (Van der Waals) and the repulsive electrical double layer forces, which are explained by the DLVO theory (Rushton et al., 2000). Brownian motion, irregular random movement of suspended particles in a liquid, occasionally brings particles close enough that their attractive forces act to join them together, which result in the formation of small aggregates. However, the result of bringing particles together may not be sufficient because spontaneous aggregation depends on the net charge of the particle. Therefore, if the particle surface is charged, repulsive forces may prevent spontaneous aggregation because energy from brownian motion is not sufficient to overcome the repulsive energy barrier. Reduction of repulsive forces among particle require intervention of chemical-coagulants which alter the surface charge of the particles to let them interact and form aggregates (Rushton et al., 2000). Coagulants are small molecular weight (MW) inorganic salts (polyions) and polymers where the primary mechanism is charge neutralization. Figure 1.1A and 1.1B show curves: (a) small negative values for Van der Waals (attraction) forces at small distances between particles, (b) the repulsion forces acting in the opposite way, and (c) the resultant of curves (a) and (b).

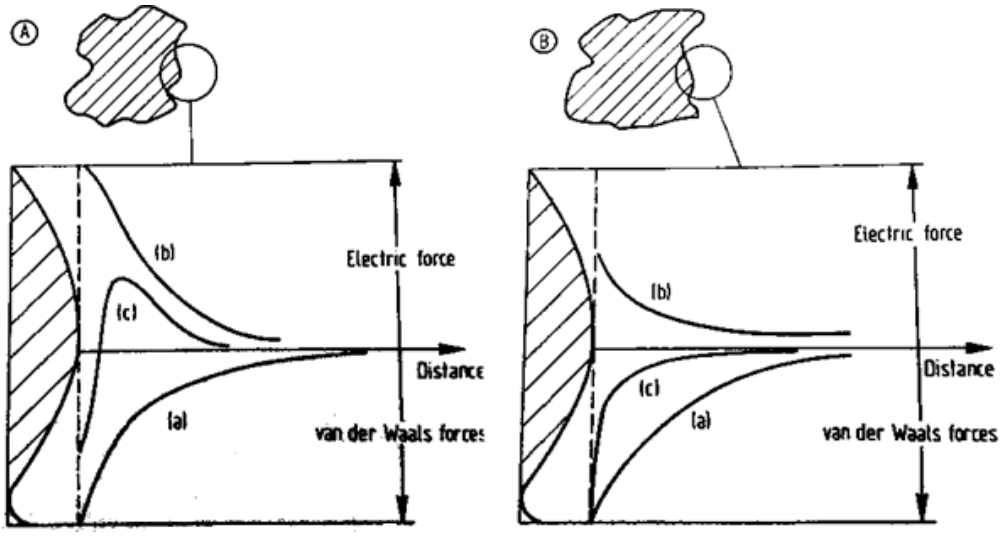

Figure 1.1. Attraction and repulsive forces of a particle in suspension before (a) and after (b) coagulant addition (Rushton et al., 2000).

1.2.2 Electric double layer and zeta potential

Electrolyte solutions containing suspended particles that have a charge associated to their surface, are likely to interact with opposite charged ions present in the suspension solution; while repelling similar ions. Hence, an electrical double layer around the particles is formed as well as a potential gradient (Rushton et al., 2000). The inner site of the double layer contains strongly bound ions (also called stern layer); while in the following layer (second layer), ions are diffused and weakly bound (diffusive layer) (Figure 1.2). The attractive forces acting between two charged particles are London and Van der Waals forces, which are opposed by the interaction of like-charges dispersed over each particle (Harrison et al., 2003). Repulsive forces must be reduced for particles to get closer; therefore, coagulants are used to decrease the repulsive forces acting

between particles so they can interact, form aggregates (flocs), and then separate from the water media.

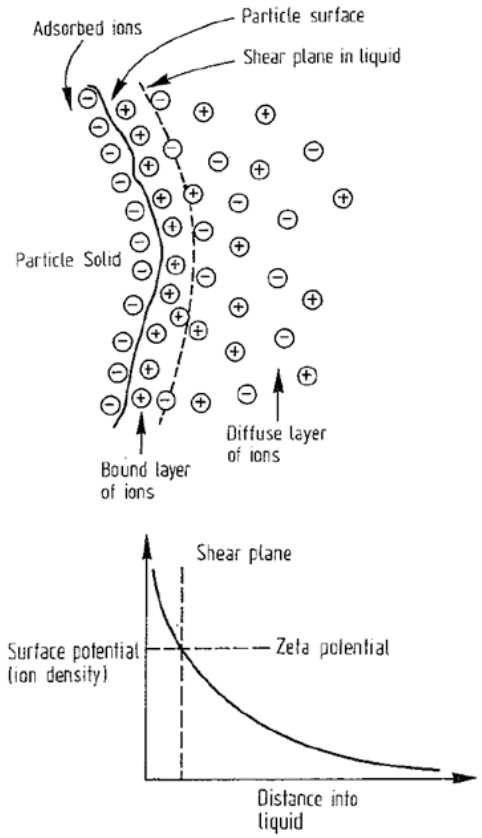

Figure 1.2. Depiction of the double layer model for charged particles suspended in an electrolyte solution (Rushton et al., 2000).

Several theories describe the electric double layer. One of the first theories was the Gouy-Chapman theory, which states that the electric potential is a function of the distance r, which is the distance from the particle surface to a uniformly distributed charge and is given by (eq. 1.1) (Harrison et al. 2003). This equation is valid for small potential values.

$$\varphi_E(r) = \varphi_{E0} EXP(-kr) \qquad \text{(eq. 1.1)}$$

where:

φ_E= Double layer potential at a distance r from the surface (mV)

φ_{E0} = Potential at the surface (mV)

k = Debye-Huckel constant (nm^{-1})

The radius of the ionic strength (Debye radius (r_{DR})), which is equal to the thickness of the double layer $(1/k)$, is a function of the concentration of dissolved ions in solution, term $\sum c_i z_i^2$ in (eq. 1.2), that is two times the ionic strength (eq. 1.3). Figure 1.3 shows the compression of the double layer when the ionic strength of the solution increases from a to b.

$$r_{DR} = \frac{1}{k} = \left(\frac{\varepsilon RT}{8\pi F^2 \sum c_i z_i^2}\right)^{1/2} \qquad \text{(eq. 1.2)}$$

$$\sum c_i z_i^2 = 2I \qquad \text{(eq. 1.3)}$$

where:

ε = Absolute dielectric constant of the liquid

R = Gas constant $\left(\frac{C^2}{J \cdot m}\right)$

T = Absolute temperature (T)

F = Faraday number $\left(9.65 \times 10^4 \frac{C^2}{J \cdot m}\right)$

c_i = Bulk concentration of different ions $\left(\frac{mol}{m^3}\right)$

z_i = Valence of different ions

I = Ionic strength of the solution (M)

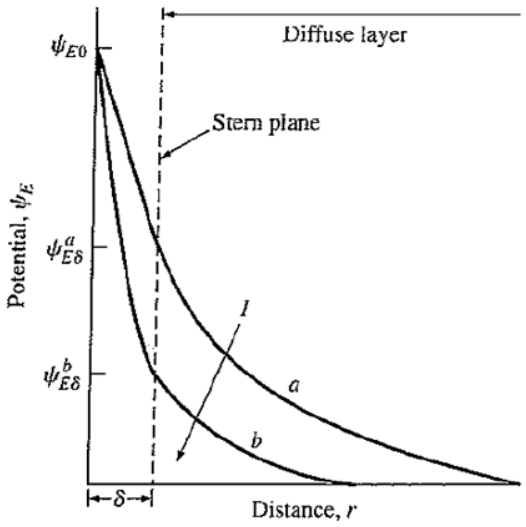

Figure 1.3. Compression of the double layer due to increasing ionic strength from curve a to curve b.

As (eq. 1.2) indicates, for small particles of low charge, the double layer is inversely proportional affected by ionic strength; therefore, the thickness of the double layer is approximately the reciprocal of the Debye Huckle function $(1/k)$. The estimated double layer thickness around a particle in sea water as well as other media is shown in Table 1.1 (Svarovsky, 1990)

Table 1.1. Double layer thickness around a particle in different media (Svarovsky, 1990).

Medium	Double layer thickness (1/k), μm
Distilled water	900
10^{-4} M NaCl	31
10^{-4} M $MgSO_4$	15
River Thames water	4
Sea water	0.4

The electrical potential formed at the surface of a particle in suspension is called zeta potential, which varies among algae species, and is sensitive to different culture media and stages of growth. These differences between algae surface charge may be explained by differences in polysaccharides that cover the surface of the particles, or in chemical composition differences among polysaccharides that cover algae cells (Knappe et al., 2004). The zeta potential serves as an indicator of the electrical state of the double layer, which is used to determine the onset of destabilization and/or charge neutralization of the system (Rushton et al., 2000).

Contrary to coagulation, the term flocculation is often used to describe aggregation of particles with large MW polymers or polyelectrolytes due to bridging mechanism rather than charge neutralization (Rushton et al., 2000) (Figure 1.4). Flocculation of particles is an electrokinetic phenomenon which depends on the overall surface charge, charge distribution, and ionic strength of the solution. The rate of aggregation involves perikinetic and orthokinetic aggregation rates (Svarovsky, 1990). Perikinetic aggregation

happens in the absence of velocity gradients; therefore, brownian motion governs aggregation rate; whilst, in orthokinetic aggregation, particle collision is enhanced by induced velocity gradients (stirring or mixing) (Svarovsky, 1990). Based on von Smoluchowski's theory, the expression for perikinetic rate is given in (eq. 1.4) and it reaches its maximum value when one particle binds to another in every collision (Svarovsky, 1990)

$$-\frac{dn}{dt} = k_p n^2 \qquad \text{(eq. 1.4)}$$

$$k_p = \frac{4kT}{3\mu} \qquad \text{(eq. 1.5)}$$

where:

n = Number of particles in a volume

k_p = Specific rate constant (mV)

μ = Viscosity of the liquid bulk $\left(\frac{g}{cm \cdot S}\right)$

k = Boltzmann's constant $\left(\frac{J}{K}\right)$

$$n = \frac{n_0}{1+k_p n_0 t} \qquad \text{(eq. 1.6)}$$

n_0 = Number of particles of radius r_0

Hence, the time to decrease the initial number of particles to its half is given by (eq. 1.7) as:

$$t_{1/2} = \frac{1}{8\pi D r n_0} \qquad \text{(eq. 1.7)}$$

where D is the diffusion constant $\left(\frac{cm}{s}\right)$ and is given by (eq. 1.8).

$$D = \frac{kT}{6\pi\mu r_0} \qquad \text{(eq. 1.8)}$$

However, brownian motion by itself is difficult to produce aggregates of an acceptable size that become flocs in a reasonable time (Svarovsky, 1990); therefore, increasing the collision rate by stirring the solution can enhance flocculation rate. This process is called orthokinetic flocculation and is governed by (eq. 1.9) (Svarovsky, 1990). From (eq. 1.9) flocs formation is directly related to the shear rate i.e., high energy shear rates can break the flocs formed during the low energy collision.

$$-\frac{dn}{dt} = \frac{2}{3} G d^3 n^2 \qquad \text{(eq. 1.9)}$$

G = Shear rate (s^{-1})

d = Diameter of the particle (nm)

Flocculation is a process in which stable suspended particles in aqueous solution are destabilized by the addition of chemicals, called flocculants, which encourage aggregation and cluster-like flocs formation (Harrison et al., 2003). The mechanism of flocculation is often difficult to distinguish from coagulation and more than one mechanism may be involved in particles agglomeration (Harrison et al., 2003). Flocculation reaction is irreversible, whilst coagulation is a reversible reaction (Rushton et al., 2000). The reduction in the zeta potential caused by the coagulants (small MW chemicals), is not an important effect when using flocculants (large MW polyelectrolytes); this hypothesis is confirmed by the high flocculation efficiencies achieved with some nonionic polymers or flocculant that carry similar charge to the particles being flocculated, which demonstrate that length of the polymer and bridging effect is sufficient for flocculation (Rushton et al., 2000). Mixing the suspension after addition of the flocculant is very important to disperse it and to increase the collision rate between particles as shown in (eq. 1.9); however, vigorous mixing can break the flocs formed and decreased flocculation efficiency.

Since this dissertation deals with aggregation of negatively charged biological particles, the term “flocculation” will be used to describe aggregation phenomenon irrespectively of the mechanism.

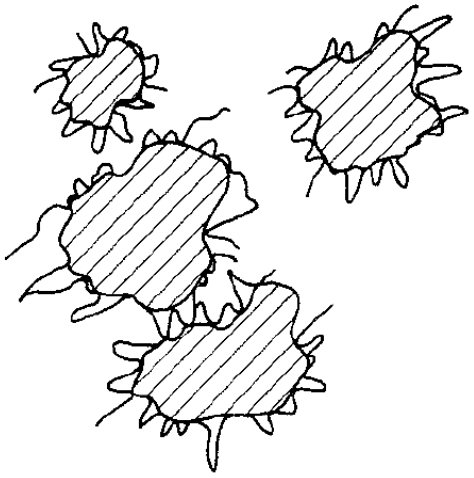

Figure 1.4. Flocculation model (Rushton et al., 2000).

1.2.3 Flocculation by inorganic electrolytes

Flocculation mechanism vary depending upon the flocculant type i.e., whether using polymers or inorganic salts. Flocculation with multivalent cations of inorganic salts is often caused by partial charge neutralization and sweep flocculation (enmeshment in a precipitate). Charge neutralization consists on reducing the repulsive forces among cells; while, sweep flocculation process is driven by the precipitation of amorphous hydroxide molecules instead of electrostatic interactions (Duan and Gregory, 2003; Garzon-Sanabria et al., 2012; Knappe et al., 2004). Charge neutralization occurs when cations such as aluminum or iron hydrolysis products or organic polymers adsorb onto the surface of negatively charged particles (Knappe et al., 2004). Furthermore, positively charged metal hydroxide precipitates also adsorb on the surface of negatively charged particles, contributing to charge neutralization; therefore, the dosage required for charge neutralization when using electrolytes, depends on the concentration of negatively

charged matter in suspension, which include particles in suspension and dissolved organic matter (Knappe et al., 2004). Natural organic matter (NOM), which consist of particles present in the suspension, is known to adsorb and increase the negatively charge associated to the surface of the particle if they are negatively charged or in the opposite case, if particles are positively charged, NOM causes charge reversal; therefore, flocculants should be added in sufficient amount to neutralize adsorbed and dissolved NOM (Knappe et al., 2004)

Autoflocculation of algal biomass does not require addition of flocculants, which makes it economically attractive. Similar to electrolyte flocculation, the mode of action of autofloculation is by increasing the pH under the presence of calcium and magnesium ions. Hydrolysis of Ca and Mg cations form CaOH and MgOH precipitates, which coagulate algae cells by sweep flocculation mechanism (Vandamme et al., 2012a; Wu et al., 2012). Calcium phosphate precipitates also work as flocculation agent. For instance, Sukenik and Shelef (1984) found that specific concentrations of calcium and orthophosphate ions in the medium are necessary before raising the pH to 8.5-9.0 for autoflocculation by calcium phosphate precipitates.

1.2.4 Flocculation by polymers

Polymer flocculation is achieved by particle bridging. Polymer chains adsorb onto the surface of a particle and then extending to nearby particles that also adsorb until an optimum floc size (cluster of particles) is formed (Rushton et al., 2000). The molecular weight of the polymer (length) is an important factor for an efficient flocculation; if the

molecular weight is too low, bridging cannot take place, and if it is too high, polymer become difficult to dissolve and disperse, i.e., synthetic polyacrylamide based polymers have an optimal molecular weight of 10^5 to 10^6 dalton (Da) (Rushton et al., 2000). Polyacrylamide residual monomer left in solution is toxic; however, its toxicity could be reduced to acceptable levels with further processing. For instance for water treatment < 0.025 % free acrylamide monomer is acceptable (Rushton et al., 2000).

Synthetic polymers used as flocculants have gained much attention since they can use different source monomers (Rushton et al., 2000). Moreover, they can be designed to be cationic, anionic, and nonionic nature, as well as different molecular weights, which expand their applications (Rushton et al., 2000).

Chitosan is a well-known natural polymer produced by alkaline deacetylation of chitin, which is a biopolymer obtained from shellfish (Bough et al., 1978; Renault et al., 2009). Chitosan has been extensively used in water treatment for removal of contaminant particles (Renault et al., 2009) and expanded as a flocculant agent for harvesting fresh and marine microalgae (Divakaran and Pillai, 2002; Renault et al., 2009; Şirin et al., 2012).

Lubian (1989) tested marine microalgae flocculation of 11 different species using chitosan as a flocculant agent. Chitosan flocculation was found to be pH sensitive for fresh and marine microalgae, i.e., some of the species required pre and post pH adjustment to enhance flocculation efficiency. Optimal pH found for fresh water species was ~ pH 7.0 , while, for marine microalgae it required pretreatment at pH 5.0 to 6.5 followed by further adjustment to pH 7.0-8.0 to precipitate it (Divakaran and Pillai,

2002; Lubián, 1989). Chitosan has the additional advantage over polyacrylamide based polymers that is environmental friendly, which expands its application to pharmaceuticals, agriculture, food processing, and nutrition (Kumirska et al., 2011; Renault et al., 2009)

1.2.5 Biofuels from microalgae

Biodiesel derived from microalgae is a potential alternative to be used as a renewable energy source (Uduman et al., 2010a). Microalgae can produce oil from sunlight and CO_2, which is also environmentally beneficial because of CO_2 sequestration (Chisti, 2007). Additionally, high quality land requirement is reduced in algae production because even marginal arid lands can be used in algae production (Uduman et al., 2010a). For instance, to replace 50 % of the total transport fuel needs in U.S using biodiesel from microalgae, 1 % to 3 % of the total cropping area would be required; while, biodiesel obtained from oil palm and corn would require 24 % to 846 % of the total cropping area respectively (Chisti, 2007; Uduman et al., 2010a).

Microalgae are a good alternative to traditional energy crops because it does not compete with food crops (Chisti, 2007), it grows continuously throughout the year (Uduman et al., 2010a), and grow faster than regular plants, i.e., doubling time usually occurs within 24 h and can be as fast as 3.5 h in the exponential phase (Chisti, 2007; Uduman et al., 2010a). Several saline and fresh water microalgae species can accumulate different lipid amounts (20 % to 50 %), carbohydrates (~ 20 %), proteins (20 % to 40

%), and other compounds (~ 10 %) of their dry weight biomass (Becker, 2007; Chen et al., 2009b; Chisti, 2007).

Microalgae are cultured in aqueous media reaching relatively low final concentrations depending on whether they grow in open ponds or in tubular photobioreactors (PBR), where they usually reach a dry weight biomass about 0.14 g/L and 4 g/L respectively; however, oil recovery and conversion into biodiesel is independent of the growth system, i.e., if the biomass is produced in a PBR or in open systems (Chisti, 2007). Open systems and PBR differ by the exposure of microalgae to the environment or growth in closed configurations, respectively. Open systems are more vulnerable to contamination, evaporative losses, CO_2 diffusion to the environment, and large area requirements to avoid light limitation issues (Pulz, 2001). On the other hand, cultivation in close systems (PBR) has the advantage to keep control of most of the parameters mentioned above. PBR are available in different shapes, sizes, and lengths, where light and mixing conditions are key factors for their success (Pulz, 2001). With regards to biomass productivity, PBR are more suitable to reach higher yields than opens systems (Pulz, 2001)

1.2.5.1 Harvesting/dewatering of microalgae by flocculation

Several factors affect harvesting of microalgae by flocculation; some of the most important factors are: flocculant dosage, cell concentration, ionic strength, algogenic organic matter (AOM), cell morphology, and media pH (Garzon-Sanabria et al., 2012; Henderson et al., 2008b; Henderson et al., 2008c; Schlesinger et al., 2012b; Sukenik and

Shelef, 1984; Uduman et al., 2010a). Optimal flocculant dosage for efficient flocculation of marine microalgae (> 90 %) was mainly affected by cell concentration and AOM (Garzon-Sanabria et al., 2012; Henderson et al., 2008b); for instance, optimal cell concentration for harvesting marine microalgae with the minimal amount of $AlCl_3$ (50 mg/L) in the absence of AOM was ~ 10^7 cells/mL (Garzon-Sanabria et al., 2012). A 10-fold increase in cell concentration (10^8 cells/mL) required 2-fold increase in flocculant dosage, while low cell concentration (10^6 cells/mL) resulted in a 6-fold increased flocculant (Garzon-Sanabria et al., 2012).

AOM comprise proteins, carbohydrates, glycollate, lipids, nucleic acids, amino acids, and small molecules (Fogg, 1983; Knappe et al., 2004). These compounds are released to the media as the cells grow, by active and passive release, autolysis, and bacterial breakdown of algae cells (Knappe et al., 2004). Release of AOM change with variations in the physiological stage of algae, which depends on the temperature, light conditions, and nutrient availability (Knappe et al., 2004). Excretion of AOM has been shown to be enhanced under exposure to bright light (Knappe et al., 2004). Accumulation of proteins and carbohydrates vary with algae species and state of growth (Bernhardt et al., 1985). Proteins and carbohydrates in the AOM have been identified as a potential problem for harvesting microalgae via flocculation and membrane filtration in water treatment; for instance, proteins are known to interfere with flocculants (Tirado-Miranda et al., 2003) and together with carbohydrates to foul membranes (Her et al., 2004). Henderson et al. (2008a) work provides clear information on how AOM affects flocculant demand on four different fresh water microalgae species; they found a

significant variation in the contribution of AOM to the flocculant demand among the species studied, which is also related to the surface charge of the cells and morphology (Henderson et al., 2008b).

Flocculation efficiency with inorganic electrolytes and polymers has been reported to be hampered by high ionic strength in the media (Bilanovic et al., 1988; Sukenik and Shelef, 1984), which up to date has limited the utilization of flocculation as a harvesting method for concentrating marine microalgae biomass. The pH of the media also plays an important role in flocculation and should be optimized depending on the chemistry of the chemical used as flocculant aid. For instance, when using aluminum or iron salts, it required acidic pH (5.0 to 6.0) (Uduman et al., 2010a); whilst, basic pH (> 10) was required for efficient flocculation with calcium or magnesium salts (Sukenik and Shelef, 1984). Polymer flocculation also required pH adjustment for optimal flocculation efficiency; for instance, when using chitosan (a natural polymer from shrimp shell) it required pre adjustment of the pH to 6.5 and then an increase to 8.0 to precipitate algae biomass. Synthetic polyacrylamide polymers also require pH adjustment to 7.0 for optimal flocculation of fresh water microalgae (Bilanovic et al., 1988).

1.2.6 Expression systems for recombinant protein production

Selection of the expression system to produce recombinant proteins is highly associated to the protein production cost, purity, integrity of the protein, and level of expression in the system chosen (Walker et al., 2005). The most common biosystems currently used to produce commercial heterologous recombinant proteins are bacterial

and yeast fermentation as well as, mammalian cell culture (Cereghino and Cregg, 1999; Schütt et al., 1997; Swartz, 2001). Bacterial and yeast fermentation has been used for long time as a major source of native (non-transgenic) proteins because of their rapid growth and economic investment production cost; however, bacterial systems fail to perform post-transcriptional and posttranslational modifications such as, intron-splicing, glycosylation and multimeric protein assembly; for instance, high molecular weight proteins cannot be produced (Cereghino and Cregg, 1999; Swartz, 2001; Walker et al., 2005). Another major disadvantages of bacterial fermentation systems is the production of proteins as insoluble inclusion bodies, the presence of endotoxins, and proteases, which complicates downstream processing (Walker et al., 2005). Even though yeast are able to perform post-translational modifications, it usually does not perform it correctly and proteins end up being hyperglycosylated (Cereghino and Cregg, 2000; Fischer et al., 1999).

Using animals as expression systems to produce high-value proteins has also been implemented; nevertheless, it is economically costly (cultivation, maintenance, and downstream processes), difficult to scale up, labor intensive, and has the potential of contamination of the protein with harmful diseases or viruses (Walker et al., 2005).

Terrestrial plants, is another way to express transgenes and it offers several advantages over bacterial, yeast, and mammalian expression systems such as, lower capital investment, ability to produce complex recombinant proteins i.e., antibodies, enzymes, vaccines, hormones among others, and they do not represent a risk as a host for human pathogens (Walker et al., 2005). However, there is a concern about transgene

flow to other plants by pollen (Mayfield *et al.*, 2007). Additionally, expression levels of recombinant proteins expressed in transgenic plants should reach > 5 % total soluble protein (TSP) to be commercially feasible (Walker et al., 2005).

Microalgae, which is a term that refers to photosynthetic microorganisms such as, prokaryotes (cyanobacteria) and eukaryotes, is currently being investigated as a target to express transgenic proteins for pharmaceutical purposes. More specifically eukaryotic microalgae such as *Chlamydomonas reinhardtii, Dunaliella salina*, *Chlorella*, *Euglena*, *Haematococcus*, and the diatoms *Phaeodactylum tricornutum*, are a few of the algae strains that have been studied as model systems to produce natural and recombinant proteins of interest (Gregory et al., 2012; Griesbeck et al., 2006; Mayfield et al., 2007; Rasala et al., 2010; Tran et al., 2012; Walker et al., 2005). Eukaryotic microalgae for recombinant protein production offer several advantages over bacterial, yeast, mammalian, and terrestrial plants systems: such as, rapid growth, easy scale up, low production cost, and no risk of transgene flow (Griesbeck et al., 2006; Mayfield and Franklin, 2005; Walker et al., 2005). The fact that one can engineer microalgae to express high-value proteins and accumulate lipid precursors for biofuels, makes microalgae an attractive vehicle for industrial enzymes and bioenergy production. However, there are currently limitations to use microalgae as protein production systems which include low and inconsistent protein yields, poorly understood complexity of cell lysates for the development of optimal recovery methods and, related to the latter, no information on purification options and potential of platform scalability.

1.2.6.1 C. reinhardtii *as an expression system for high-value recombinant proteins*

C. reinhardtii is a unicellular eukaryotic green microalgae that is being used as a model system to express different recombinant proteins of industrial as well as pharmaceutical applications because of its good standing genetic characteristics and ease of transformation (Mayfield and Schultz, 2004). *C. reinhardtii* can express transgenic proteins in three different ways: 1) expression of the transgene in the nucleus and targeting the protein to the cytoplasm, 2) expression of the transgene in the nucleus and secretion via endoplasmatic reticulum and Golgi apparatus, and 3) expression of recombinant protein and accumulation within the chloroplast. (Figure 1.5) (Griesbeck et al., 2006).

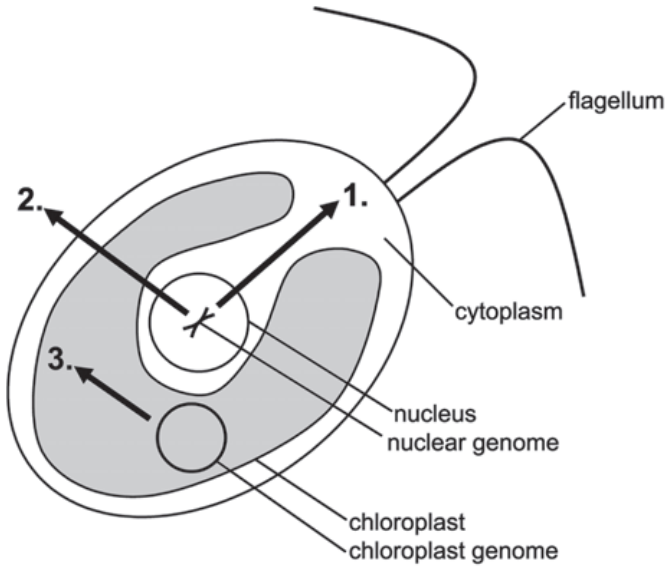

Figure 1.5. Different compartments of transgene expression in *C. reinhardtii* (Griesbeck et al., 2006).

Gene expression in the nucleus is still under development because further optimization is required (Eichler-Stahlberg et al., 2009). Silencing of transgenes via transcriptional and posttranscriptional mechanisms is a big disadvantage of randomly inserted transgenes into the nuclear genome (Griesbeck et al., 2006). Proteins that require glycosylation for proper folding and biological activity might not express efficiently in the chloroplast because the latter does not have endoplasmatic reticulum (ER) and Golgi apparatus. Limited information of protein production in the chloroplast of *C. reinhardtii* is available (Mayfield and Franklin, 2005; Rasala et al., 2010). Some of the first attempts to express recombinant proteins in the chloroplast of *C. reinhardtii* under the control of promoters such as: *rbcL, psbA,* and *atpA* resulted in none or very low accumulation of protein (Blowers et al., 1989; Blowers et al., 1990; Ishikura et al., 1999); therefore, further studies demonstrated that to achieve successful recombinant protein accumulation in *C. reinhardtii* chloroplast it requires codon optimization and also 5' and 3' untranslated regions (UTR) (Mayfield et al., 2003; Mayfield et al., 2007; Mayfield and Schultz, 2004; Tran et al., 2009).

C. reinhardtii chloroplast has been evaluated for difficult to express and assemble protein molecules, because of its ability to proper fold this type of molecules by using its endogenous protein disulfide isomerases and complex chaperones. A fusion protein containing the cholera toxin B attached to the foot and mouth disease virus (FMDV) VPI gene was expressed to a level of 3 % TSP in the chloroplast of *C. reinhardti* to be used as a vaccine source (Sun et al., 2003). Furthermore, a construct containing the coding sequence for a human protein tumor necrosis factor-related apoptosis inducing ligand

(TRAIL) was also expressed in the chloroplast of *C. reinhardtii* and produced at a level of 0.43 % to 0.67 % TSP, this recombinant protein is gaining a lot of attention for cancer treatment because of its killing specificity; (Yang et al., 2006).

About 40 different recombinant proteins including human antibodies, hormones, antibody fragments, and enzymes have been successfully expressed in the eukaryotic algae chloroplast at levels between 0.5 and 1 % TSP (Mayfield et al., 2007; Tran et al., 2009). A full-length IgG1 human monoclonal antibody against anthrax protective antigen 83 (PA83) was successfully expressed and assembled in *C. reinhardtii* transgenic chloroplast (Tran et al., 2009) indicating that *C. reinhardtii* chloroplast is able to produce complex proteins. In 2010, Rasala et al. (2010) selected seven proteins from a wide range of human therapeutics for expression in the chloroplast genome to demonstrate *Chlamydomonas* versatility as an expression system. Half of the tested proteins were produced at greater than 1 % TSP, one protein could not be detected by western blot and the other two were detected at low level. Protein instability and poor translation of chimeric mRNA have been a potential explanation for the poor expression (Coragliotti et al., 2011; Surzycki et al., 2009). Surzycki et al. 2009 analyzed factors affecting protein expression in *C. reinhardtii* chloroplast and listed the following main factors that require attention for optimal performance: codon optimization, recombinant protein toxicity, host protease activities, and transformation–associated genotypic modification. Strategies for increasing protein expression levels and recent improvements in this area have been summarized by (Potvin and Zhang, 2010). Despite of the recent progress and continuing development of transgenic algal biotechnology,

there is no consensus about methodology and strategies for obtaining high-level protein accumulation and, even less so, optimal isolation and purification of extracted protein products.

1.2.6.2 Extraction and recovery methods used for extraction of recombinant proteins from Chlamydomonas

Several cell disruption techniques for extraction of intracellular enzymes and proteins into solution are available. They are classified as gentle, moderate, and vigorous techniques that should be selected depending on the type of tissue to be treated (Scopes, 1982). For instance, animal and plant cells disruption technique vary due of the difference in cell wall composition, i.e., plants cell wall is more rigid because of its cellulosic composition (Scopes, 1982). Efficient cell disruption must be accomplished to maximize recombinant protein released; therefore, physical disruption of aqueous culture biomass such as lemma, plant cells, and microalgae can be achieved using high-shear mixers or homogenizers in a biomass:buffer ratio between 1:1.5 to 1:8 (Wilken and Nikolov, 2012). Extraction of recombinant proteins from leafy tissue is accompanied by other water-soluble components such as DNA, polysaccharides, alkaloids, phenolics, proteases, and pigments (chlorophyll-derived), which impose additional challenges in downstream processing, and ultimately affect quality and quantity of target proteins (Barros et al., 2011; Wilken and Nikolov, 2012). Likewise, microalgae extracts, deal with chlorophyll-derived pigments, proteases, polysaccharides, and other algogenic

organic matter (AOM). Therefore, recombinant proteins extraction procedures used for leafy tissue could be applied to microalgae as both systems faced similar challenges.

Optimization of extraction conditions for maximal recombinant protein released and to reduce interaction with indigenous proteins, pigments, and other compounds present in the extract, require evaluation of buffer composition, disruption technique, tissue to buffer ratio, and target protein organelle expression (Wilken and Nikolov, 2012; Zhang et al., 2005). Additionally, protection of recombinant protein from degradation during extraction is important to decrease purification cost. For this purpose, control of extract pH, temperature, and extraction time, as well as protein stabilizers such as: protease inhibitor, antioxidant, and metal chelating agents, are added to the extraction buffers based on the type of proteases identified (Wilken and Nikolov, 2012). Furthermore, the ratio of recombinant to native proteins can be controlled adjusting the extraction pH and ionic strength (Wilken and Nikolov, 2012); hence, addition of low NaCl (200-500 mM) concentration to the extraction buffer is usually found.

Proteins expressed in chloroplasts seem to require detergents to reduce protein interaction with thylakoid membrane (Daniell et al., 2009; Tran et al., 2009). Detergents are frequently used for partial destruction of cell wall in plants. Nonionic detergents such as, Triton X-100, Polyoxyethylene*p*-*t*-octyl phenol, octylβ-glucoside, and Tween 20 (PEG-20 sorbitanmonolaurate) are less denaturing agents for proteins present in the extract than ionic detergents, such as dodecyl sulfate (Harrison et al., 2003). Nonionic detergents are used at low concentrations not to harm extracted proteins.

Buffer selection for protein extraction should consider solubility of the proteins in the liquid phase, a pH that does not cause protein denaturation, and in the case of plants, a detergent that help with cell wall disruption but at the same time does not harm extracted proteins (Harrison et al., 2003). Furthermore, buffer composition should not be complex so that reagent cost is not an issue and also the presence of some particular compounds does not interfere with the product recovery step (Wilken and Nikolov, 2012). Frequently buffers used for extraction of recombinant proteins from green tissue are phosphate buffer for basic pH extraction, acetate buffer for acidic pH extraction, and Tris buffer for neutral pH (Barros et al., 2011; Tran et al., 2009; Zhang et al., 2005). Clarification of plant cell cultures and algae are usually performed by centrifugation followed by depth filtration (Wilken and Nikolov, 2012).

1.2.6.3 Purification of recombinant proteins expressed in C. reinhardtii

Limited information on purification of recombinant protein expressed in *C. reinhardtii* is available; the majority of recombinant proteins expressed in the chloroplast of microalgae have been purified by FLAG affinity chromatography because they contain the immunoaffinity FLAG-tag for easy of detection and purification (Gregory et al., 2012; Mayfield et al., 2003; Rasala et al., 2010; Tran et al., 2012). For instance, the two proteins studied in this dissertation, immunotoxin (MT51) and Pfs25 antigen, have been purified using anti-FLAG affinity resin (Gregory et al., 2012; Tran et al., 2012). Binding of these recombinant proteins as well as other FLAG-tagged proteins to the affinity resin has been done in batch mode by shaking the affinity resin and clarified

extracts for 2-4 hours at 4^{o}C; adsorbed recombinant proteins were eluted right after incubation with the affinity resin (Gregory et al., 2012; Tran et al., 2012), or after overnight incubation using elution buffer at pH ~3.5 (Rasala *et al.*, 2010). This purification procedure is clearly not scalable and slow binding and elution kinetics (4 h and > 8 h respectively) indicate the need for thorough analysis of clarified algal homogenates, to identify the factors that affect binding/elution kinetics and purification yield.

Purification of a full monoclonal antibodies expressed in *C. reinhardtii* has been successfully achieved using Protein G resin, which binds to the Fc domain of the antibody. For instance, the full-length IgG1 human monoclonal antibody against anthrax protective antigen 83 (PA83) was purified by affinity chromatography with Protein G resin (Tran et al., 2012). Most of the affinity purified proteins contained minor degradation products but the origin of degradation (intracellular or extracellular) has not been discussed. Further purification to remove degradation products has been done by size exclusion chromatography (Tran et al., 2012; Tran et al., 2009).

1.3 Objectives

This dissertation seeks to understand the main factors that are limiting harvesting/dewatering of marine microalgae via flocculation to be used as a platform for lipid production for the biofuel industry, and expression of high-value recombinant proteins for therapeutic applications. This investigation addresses two different industrial bioprocessing technology challenges and applications: 1) recovery of algal biomass by flocculation and sedimentation for energy purposes and 2) recovery and purification of two high-value recombinant proteins for therapeutic application. These two bioprocessing projects are closely related from a downstream processing view point as both address separation challenges and concentration unit operations. The overall goal of this dissertation was to understand and identify potential barriers to develop downstream processes using green microalgae as a platform for biofuel production and high-value recombinant proteins

Specific objectives regarding harvesting of marine microalgae intended for biofuel applications are:

1. To understand flocculation mechanism with inorganic electrolyte i.e., $AlCl_3$

2. To identify the effect of process variables such as, cell concentration, ionic strength, coagulant dosage, media pH, and cell surface charge, affecting harvesting efficiency of marine microalgae with inorganic electrolyte ($AlCl_3$)
3. To evaluate the effect of polymer molecular weight and charge density, algogenic organic matter (AOM), and salt concentration on harvesting efficiency of marine microalgae
4. To estimate cost analysis of different types of flocculants, i.e., inorganic salt, natural and synthetic polymers

Specific objectives dealing with expression of recombinant proteins in *C. reinhardtii* chloroplast are:

1. To understand and select cultivation conditions for maximal biomass production of *C. reinhardtii*
2. To evaluate induced expression of recombinant proteins produced in the chloroplast of *C. reinhardtii* by light exposure time

1.4. Dissertation organization

This dissertation is organized by chapters. Each chapter begins with a summary that introduces the reader to the main findings of the work accomplished in that particular chapter. A detailed introduction including previous work and the purpose of the study is presented. Chapters II, III and IV continue with a description of the materials and methods used for the development of the study followed by the presentation of results and discussion where the main findings are remarked.

Chapter I: entitled "Introduction" contains a general introduction of the work presented in this dissertation, where the focus of this work is emphasized, followed by a literature review that covers basic concepts and background of the topics studied. Objectives and dissertation organization, which describes how this work is organized, are also included in this first chapter.

Chapter II: entitled "Harvesting *Nannochloris oculata* by inorganic electrolyte flocculation: effect of initial cell concentration, ionic strength, coagulant dosage, and media pH" covers a detailed literature review on flocculation with inorganic salts, flocculation mechanism, and the most influencing variables affecting flocculation efficiency. This chapter focuses on identifying the most influencing variables affecting marine microalgae flocculation with an inorganic salt.

Chapter III: entitled "Effect of algogenic organic matter (AOM) and sodium chloride on *Nannochloropsis salina* flocculation efficiency", includes a literature review on polymer flocculation of marine microalgae as well as the effect of algogenic organic matter (AOM) in flocculation efficiency. This chapter examines the effect of polymer

molecular weight and charge density, AOM, and salt concentration on harvesting efficiency of marine microalgae.

Chapter IV: entitled “Optimizing induced expression of recombinant proteins driven by the *psbA* promoter in the chloroplast of *C. reinhardtii*” consists of a literature review on cultivation of microalgae and bioreactors, *C. reinhardtii* as a model system for expression of complex recombinant proteins, and a description of the two constructs for the recombinant proteins herein studied. This chapter focuses on selecting growth conditions for optimal biomass production of transgenic *C. reinhardtii* and induced transgene expression by light exposure.

Finally, chapter V: entitle “Conclusions and recomendations” state the main findings obtained with the development of this study and recommendations for future work.

CHAPTER II

HARVESTING *Nannochloris oculata* BY INORGANIC ELECTROLYTE FLOCCULATION: EFFECT OF INITIAL CELL CONCENTRATION, IONIC STRENGTH, COAGULANT DOSAGE, AND MEDIA PH*

2.1 Overview

Process variables affecting harvesting efficiency of *Nannochloris oculata* by $AlCl_3$ flocculation such as, cell concentration, ionic strength, coagulant dosage, media pH, and cell surface charge were investigated. Initial cell concentration and coagulant dosage had a significant effect on the removal efficiency; however, levels of ionic strength tested were not significant. Best flocculation conditions of investigated variables were: 0.0016 ng of $AlCl_3$/cell, 3.0×10^7 cell/mL, and pH 5.3. Removal efficiency at optimum conditions and salt concentrations of: 0, 15, and 30 g/L NaCl was 96, 98, and 97 %, respectively. Low cell concentration cultures ~10^6 cell/mL, required five times greater $AlCl_3$ dosage to achieve the same removal efficiency. Destabilization of algal cultures using 0.0032 ng of $AlCl_3$/cell was observed by reducing the zeta potential to -22 mV. Acidification with HCl for conducting flocculation at pH 5.3 could be a significant cost burden unless is mitigated by selecting a low-buffering-capacity media.

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*Reprinted from Andrea J. Garzon-Sanabria, Ryan T Davis, Zivko L. Nikolov, Harvesting *Nannochloris oculata* by inorganic electrolyte flocculation: Effect of initial cell density, ionic strength, coagulant dosage, and media pH, Bioresource Technology, 118, 418-424, Copyright 2012, with permission from Elsevier.

2.2 Introduction

Microalgae have a variety of potential applications ranging from industrial food and feed products to biofuels (Becker, 2007; Chisti, 2007; Molina Grima et al., 2003; Uduman et al., 2010a). The use of algae for biofuel production has a great potential because algae can sequester CO_2 and capture solarenergy10-50 times more efficiently than terrestrial plants (Lam and Lee, 2012). In addition, algae cultivation does not compete with food crops because algae can use arid lands that are not suitable for crops (Chisti, 2007; Uduman et al., 2010a).

Microalgae cultures have relatively low final concentrations and depending on whether they are grown in open ponds or tubular photobioreactors (PBR) could reach 0.5 to 5g/L dry weight (Lam and Lee, 2012). Low biomass concentrations and the small size of microalgae pose a significant harvesting challenge. Recent lifecycle analysis reported by Sander and Murthy (2010) indicate that concentration (bulk harvesting) of algal biomass by centrifugation or filtration is energy and maintenance intensive. Pre-concentration of dilute algal cultures by inorganic coagulants is currently considered as the most promising option for harvesting because flocculation is easily scalable and can be applied to a wide range of algal species (Uduman et al.,2010).

Previous flocculation studies with inorganic salts demonstrated that algal flocculation could be induced under alkaline or acidic conditions. Algal flocculation in alkaline conditions (pH > 10) was induced by precipitation of calcium (Ca) and /or magnesium (Mg) salts present in the media (Sukenik and Shelef, 1984), while inorganic flocculants such as aluminum sulfate and iron chloride required acidic pH for optimal

algal flocculation (Uduman et al., 2010). Microalgal flocculation at high pH has been recently re-examined to show that $Ca(OH)_2$ and/or $Mg(OH)_2$hydroxide are involved in the flocculation process although, the underlying mechanism and the role of these cations remains uncertain (Wu et al., 2012). Wu et al. (2012) tested flocculation of three freshwater and two marine species in the presence of magnesium salts present in the media. They concluded that hydrolysis of Mg^{2+} in the growth media formed $Mg(OH)_2$ precipitate at high pH, which induced flocculation of algal cells by sweep flocculation and charge neutralization. Smith and Davis (2012) and Vandamme et al. (2012a) demonstrated that flocculation of freshwater algae (*Chlorella vulgaris*) could be induced at pH >10 and the presence of Mg^{2+}in the growth medium was essential to induce flocculation. Additional $Mg(OH)_2$ increased flocculation efficiency but reduced sedimentation rates due to excessive precipitate bridging and hindered settling (Smith and Davis, 2012). A different conclusion was reached by Schlesinger et al.(2012a) who tested nine different freshwater and marine algae species to show that $Ca(OH)_2$, NaOH, KOH, and NH_4OH could induce flocculation, but $Mg(OH)_2$ could not. The best flocculation efficiencies for all nine species were achieved by using $Ca(OH)_2$.

As mentioned before, aluminum and iron coagulants work more effectively in acidic conditions. Most of the current knowledge about microalgae removal using aluminum flocculants is primarily derived from water treatment studies using freshwater algae (Carlson et al., 2000; Franceschi et al., 2002; Gregor et al., 1997), although several groups have also considered algal harvesting aspects (Carlson et al., 2000; Chen et al., 2009a; Edzwald, 1993; Letterman, 1999; Meghzili et al., 2008; Uduman et al., 2010a).

The mechanism and efficiency of flocculation with hydrolyzed Al^{3+} is complex and depends on formed aluminum species and solubility of aluminum hydroxide at a particular pH (Amirtharajah and Mills, 1982; Duan and Gregory, 2003; Zhang et al., 2004). Flocculation of algal species in the range of pH 5.0 to 6.0 has been explained by the combined action of positively charged polyaluminum species and amorphous aluminum hydroxide precipitate. The contribution of amorphous precipitate to particle flocculation by entrapment rather than electrostatic interactions is referred to as "sweep flocculation".

Henderson et al. (2008a) examined flocculation of freshwater algae (green algae, blue-green algae, and diatoms) for water treatment and concluded that key properties that affected algal flocculation include cell morphology, surface charge density, cell concentration, mineral content, extracellular organic matter (EOM) concentration and composition. A study by the same group (Henderson et al., 2008b) showed that freshwater algae systems, which consist of algae cells and EOM, can be destabilized and flocculated by adjusting aluminum dosage (ng/cell) and culture pH for *Chlorella* and *Cyclotella* strains. According to this study, complete charge neutralization was not necessary to initiate cell aggregation, and the reduction of the zeta potential to at least -15 mV was sufficient for algae removal. The data from Henderson's studies (2008a, b) indicate that both charge neutralization and sweep flocculation may play a role in the flocculation process. Subsequent work demonstrated that secreted algogenic material can sterically interfere with the aggregation process and complexes with aluminum sulfate coagulant (Henderson et al., 2010).

Regarding the effect of ionic strength on flocculation, Sukenik et al., (1988) studies with marine algae found that increasing the ionic strength of the media up to 36 g/L salinity significantly decreased flocculation efficiency when using aluminum sulfate and ferric chloride. The optimal dosage for flocculating marine microalgae (*Isochryses galbana*) was 5- to 10-fold greater than that for freshwater microalgae *(C. vulgaris*). Reduction of ionic strength of *Isochryses galbana* cultures to 0.1 M by dilution with fresh media reduced the required flocculant dosage almost five-fold. This conclusion seems contrary to the expected enhancement of particle (algae) aggregation at high ionic strengths due to reduction of electric double layer (Letterman 1999).

From these studies, several important observations relevant to the objective of our work can be made. Contrary to the linear surface area correlation proposed by Henderson et al. (2008a), Schlesinger et al. (2012a) found that the required flocculant dosage was not proportional to cell concentration. The flocculation of algae cultures at cell concentrations of 10^7 and 10^8 cells/mL required 3- and 6-fold more $Ca(OH)_2$, respectively, compared with the same cultures diluted to 10^6 cells/mL. Ionic strength negatively affected the aluminum-sulfate-induced flocculation of marine algae (Sukenik et al., 1988). On the other hand, the flocculation efficiency of freshwater and marine algae using $Ca(OH)_2$ was not different (Schlesinger et al., 2012a). The data of Wu et al. (2012)show that substantially greater amount of Mg^{2+}(g/L) was removed from the media during flocculation of marine species than freshwater ones, suggesting that marine cultures used more $Mg(OH)_2$ for flocculation than the freshwater cultures.

The objective of the present study was to examine the effect of key process variables (pH, ionic strength, initial cell concentration, and Al^{3+} concentration) on flocculation efficiency of *Nannochloris oculata* (marine algae). Specifically, we wanted to address some of apparent discrepancies regarding the impact of ionic strength and cell concentration on coagulant dosage, the latter being one of the main harvesting cost drivers. To eliminate potential interference of EOM due to interaction with the coagulant, flocculation experiments with $AlCl_3$ were performed in the absence of EOM.

2.3 Materials and methods

2.3.1 Algae growth and culturing conditions

Nannochloris oculata, a green microalgae species was selected for harvesting studies because of its ability for lipid production at ~ 40% of its dry weight biomass. This species was initially obtained from Texas AgriLife Research Station at Pecos. Initial inoculums (10^5 – 10^6 cells/mL) were used to (scale up) expand the cultures from 2 L flask up to 10 L carboys. *N. oculata* was grown in a modified Erdshreiber media (The Culture Collection of Algae, University of Texas at Austin), enriched with 30 mg/L KH_2PO_4 and 15 g/L $NaHCO_3$. The cultures were grown at room temperature (25 °C) and 24 h illumination was provided by fluorescent light at 100 PPF (μmol/(m^2·s)) until they reached stationary phase (>10^7cells/mL). The pH was maintained close to 9.0 by daily addition of pure CO_2. Mixing was provided by sparging sterile air (0.2 μm filter) continuously into the cultures using aquarium pumps.

N. oculata growth curve was developed to correlate the optical density and cell count with the stage of growth. Two independent cultures of *N. oculata* algae strain were inoculated with an initial inoculum of 1.0×10^4 cells/mL from an original culture of $O.D_{750\ nm} = 0.8$. Those cultures were grown for three weeks under the conditions described above. Cell concentration (growth) was monitored daily by measuring the optical density at 750 nm wavelength using a Beckman Coulter (Brea, CA) DU640 spectrophotometer. Cells were counted using a hemocytometer (BrightLine, Hausser Scientific, Horsham, PA). All the flocculation experiments were performed when the cultures reached an $OD_{750nm\sim}$ 4.0 , which corresponds to a cell concentration of 3.0×10^7 cell/mL and ash free dry weight (AFDW) of 2.3 g/L ± 0.02.

2.3.2 Experimental design for screening flocculation variables

A 2^3 full factorial design was performed as a screening experiment to determine the effect of the flocculant type, dosage of flocculant, and media pH on harvesting efficiency. Coagulant type, pH, and dosage were tested at two levels each: i) pH: 5.0 and 7.0, ii) coagulant: $AlCl_3$ and $Al_2(SO_4)_3$, and iii) dosage of coagulant: 50 mg/L and 100 mg/L of each coagulant. Samples were randomized and two replicates at each level were performed.

2.3.3 Flocculation experiments

To eliminate the potential EOM effect on flocculant dosage and sedimentation efficiency, *N. oculata* cultures were gently centrifuged at 3000 × g for 6 min and cell

pellets resuspended in fresh media at the predetermined ionic strength and cell concentration. Fifty milliliter samples were pH adjusted using 5 M HCl, followed by the addition of the predetermined aliquots of $AlCl_3$ stock solution (40 g/L) in 100 mL glass beakers and mixed with a magnetic stir plate. The culture was mixed for 2 min at 500 rpm, followed by a slow mixing for 15 min at 60 rpm to promote aggregation. The pH was kept constant at ~ 5.3 by adding acid when needed and at the end of the 17 min period the 50 mL samples were transferred to a 50 mL gravimetric cylinder. Optical density at 750 nm was periodically measured for a 1 hour period at 5 cm below the top of the gravimetric cylinder to monitor algal settling. Flocculation experiments were performed in triplicates.

2.3.3.1 Zeta potential analysis as a function of pH and coagulant dosage

Understanding algae cell surface charge provides critical information to improve the harvesting efficiency process. Zeta potential experiments were conducted to investigate the effect of pH on surface charge of algae in suspension in the presence and absence of flocculant. A zeta potential curve vs. pH was constructed using 50 mL of *N. oculata* sample with an initial cell concentration of 1.0×10^6 cells/mL. The samples were pH adjusted from pH 7.0 to 4.0 at pH increments of 1 unit using 5M hydrochloric acid (HCl). $AlCl_3$ was added to each sample to a final dosage of 0.016 ng/cell. The other samples without $AlCl_3$ were used as control. Samples were continuously mixed on a stirrer plate at 500 rpm for 2 min followed by 15 min at 60 rpm. Afterward, 1 mL aliquots were taken and injected in the cuvette of Malvern ZetaSizer (Malvern

instruments, UK).Two replicates were performed with three repeated measurements of zeta potential at each pH tested and the average values were reported.

2.3.3.2 Zeta potential analysis when varying coagulant dosage

Zeta potential measurements of samples at two different $AlCl_3$ dosages (0.0032 ng/cell and 0.016 ng/cell) and pH5.0 were run in order to understand particle destabilization and charge neutralization phenomena. The experiments were performed using 50 mL of *N. oculata culture* with an initial cell concentration of 1.0×10^6 cells/mL by diluting the original culture with fresh media. Culture pH was adjusted down to 5.0 using 5 M HCl. $AlCl_3$ was added to the first sample to a final dosage of 0.0032 ng/cells and to the second sample to a final dosage of 0.016 ng/cell. A third sample was prepared without $AlCl_3$ to use as control. Samples were continuously mixed on a stir plate at 500 rpm for 2 min followed by 15 min of mixing at 60 rpm. At the end of the mixing period, a 1 mL aliquot was injected in the cuvette of the Malvern ZetaSizer (Malvern instruments, UK) to measure the zeta potential. Two independent samples for each dosage were run in triplicates at each flocculant dosage tested. The reported zeta potential measurements are the average of the triplicates.

2.3.3.3 Effect of initial cell concentration, ionic strength, and flocculant dosage on harvesting efficiency

Samples prepared at three different initial cell concentrations (4.0×10^6 cells/mL, 3.0×10^7 cells/mL, and 1.0×10^8 cells/mL) were used to determine the effect of cell

concentration on harvesting efficiency at three coagulant dosages and three ionic strength levels. The ratio of $AlCl_3$ to cell concentration (ng $AlCl_3$/cell) at the three initial cell concentrations was kept constant. Flocculant dosages tested were: 0.0016 ng $AlCl_3$/cell, 0.0032 ng $AlCl_3$/cell, and 0.01 ng $AlCl_3$/cell; which were equivalent to 50 mg $AlCl_3$/L, 100 mg $AlCl_3$/L, and 300 mg $AlCl_3$/L, at an initial cell concentration of 3.0×10^7 cells/mL. The effect of ionic strength was tested by resuspending algae cells in fresh media at three different NaCl concentrations (0 g/L, 15 g/L, and 30 g/L). $AlCl_3$ flocculation was performed using the same protocol described in Section 2.3.3

2.3.3.4 Removal efficiency, concentration factor, and sedimentation rate determination

Algae biomass settling took place in a 50 mL gravimetric cylinder for one hour. Harvesting efficiency was calculated using the optical density taken 5 cm below the top of the gravimetric cylinder at time zero and after 1 h settling. Algae removal efficiency (RE) was calculated by measuring optical density and replacing those values in (eq. 2.1)

$$RE = \left[1 - \left(\frac{O.D_f}{O.D_0}\right)\right] * 100 \qquad \text{(eq. 2.1)}$$

where, OD_f is the optical density after 1 h settling and OD_0 is the initial optical density at time zero.

The concentration factor (CF) provides a quantification measurement of how many fold algae cells were concentrated from an initial volume of 50 mL to a final settled

volume after 1 h. The concentration factor was calculated by Lee et al., (2009), see (eq. 2.2):

$$CF = \left(V_0 / V_f \right) \cdot (RE) \qquad \text{(eq. 2.2)}$$

where, CF is the concentration factor, V_o is the initial volume (mL) of culture used for electrolyte flocculation, V_f is the final volume (mL) occupied by the algae flocs after 1 h (at the end of flocculation period). Apparent sedimentation rates under gravity were estimated by measuring the settling distance of the flocculated algae front over time (after a period of 5 min).

2.4 Results and discussion

2.4.1 Screening experiments for optimal inorganic coagulant type, dosage and pH conditions

Previous studies showed that alum [$Al_2(SO_4)_3$] and $AlCl_3$ could be used for algae flocculation, and that their efficacy was dependent on culture pH. The most common pH used in water treatment applications ranged from pH 5.0 to 7.0. A randomized experimental design was performed to compare the removal efficiency of the two inorganic coagulants at pH 5.0 and 7.0. $AlCl_3$ at pH 5.0 was more efficient for harvesting *N. oculata* algae strain than alum (P-value for type of coagulant: 0.0002 and P-value for pH: 0.0006, respectively). The removal efficiency with $AlCl_3$ at both dosage levels (50

mg/L and 100 mg/L) was 35- and 24-fold greater than the removal efficiency achieved with alum and no flocculation was observed with either electrolyte at pH 7.0. The use of $AlCl_3$ is also a better choice than $Al_2(SO_4)_3$ because it does not introduce SO_4 anion as another variable (Duan and Gregory, 2003)

The pH effect on charge reduction and/or neutralization of *N. oculata* cultures at four different pH values and at constant coagulant dosage of 0.016 ng $AlCl_3$/cell was measured by the zeta potential of the algal colloid suspension (Figure 2.1). The data in Figure 2.1 indicate that complete charge neutralization occurred (0 mV) at around pH 5.3, and a charge reversal at pH 5.0. The control samples without coagulant did not exhibit a significant zeta potential change in this pH range. At pH 4.0 and 6.0, a charge reduction of ~10 mV compared to the control was accomplished indicating that concentration of positively charged aluminum hydroxide species at these two pHs was not sufficient to achieve charge neutralization of *N. oculata* (Amirtharajah and Mills, 1982). The zeta potential values in Figure 2.1 apparently concur with previously made observations that the pH range of 5.3 to5.6 was optimal for algae flocculation by aluminum sulfate (Becker, 1994; McGarry and Tongkasame, 1971). Based on these data, we selected pH 5.3 as optimal culture acidity for studying flocculation with $AlCl_3$.

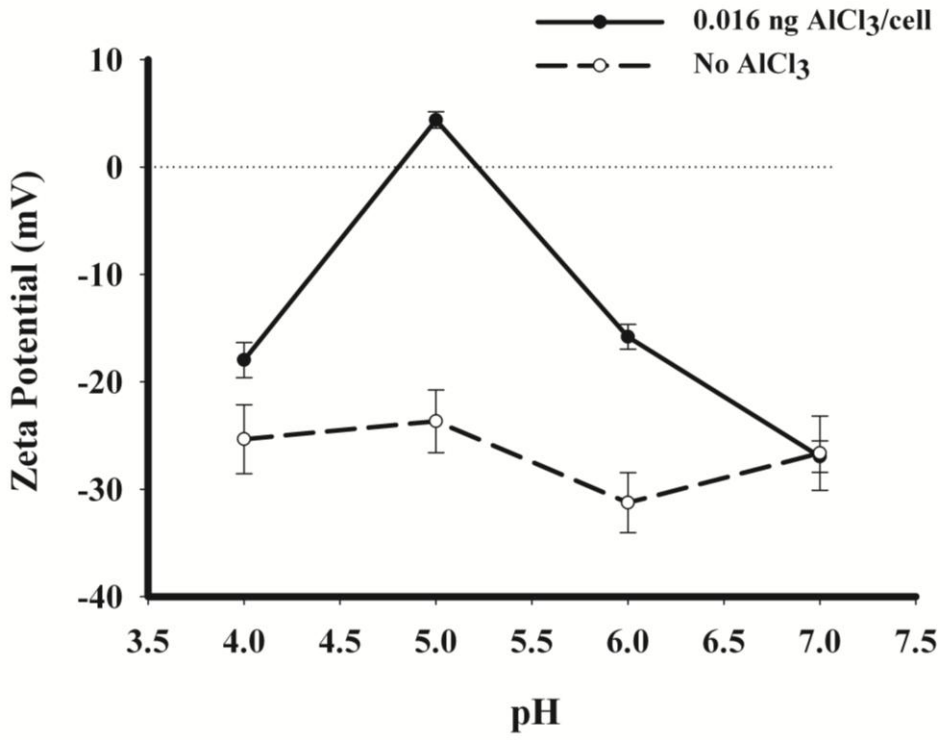

Figure 2.1. Effect of pH on zeta potential. Measurements of zeta potential of algal suspension containing 0.016 ng $AlCl_3$/cell at different pHs was measured and compared with the zeta potential of algal suspension without coagulant. The initial cell concentration of the culture was 1.0×10^6 cells/mL. Bars indicate deviation from the average of two replicates.

Having found the pH where charge neutralization of *N. oculata* algae occurred, we wanted to determine the minimum amount of coagulant required to achieve > 95 % removal efficiency. A wide range of coagulant dosages from 0.0008 ng $AlCl_3$/cell to 0.016 ng $AlCl_3$/cell, corresponding to 0.025 g/L $AlCl_3$ and 0.5 g/L respectively were tested using a cell concentration of 3.0×10^7 cells/mL and pH of 5.3.

Figure 2.2 shows a constant algae removal efficiency of > 95 % when using $AlCl_3$ concentrations between 0.016 ng $AlCl_3$/cell to 0.0016 ng $AlCl_3$/cell; however when the dosage of coagulant was decreased to 0.0008 ng $AlCl_3$/cell, there was no significant algae removal efficiency, indicating that the minimum amount of $AlCl_3$/cell that could be used to flocculate *N. oculata* established with this study was 0.0016 ng $AlCl_3$/cell. Once the coagulant was added, it was allowed to settle for 1 hour. A greater than 90 % removal efficacy achieved after 5 min settling at a coagulant dosage of 50 mg/L or 0.0016 ng/cell is also a good indicator of achievable settling rates (> 2 cm/min or 120 cm/h). Concentration factor was highest at 0.0016 ng/cell and slowly decreased with the increasing coagulant concentration. The lowest concentration factor and settling rate was observed at a coagulant concentration of 500 mg/L (0.016 ng/cell), which was the result of precipitation of insoluble aluminum hydroxide formed by aluminum hydrolysis between pH 5.0 and 6.0 (Duan and Gregory, 2003). We confirmed the formation of $Al(OH)_3$ precipitate in *N. oculata* medium and found that the precipitated mass was proportional to the initial coagulant concentration and amounted to 30 % of settled *N. oculata* volume. Best flocculation conditions found with this screening experiment were: coagulant dosage of 0.05g/L $AlCl_3$ (0.0016 ng $AlCl_3$/cell) at pH 5.3, which achieved a concentration factor of 18-fold.

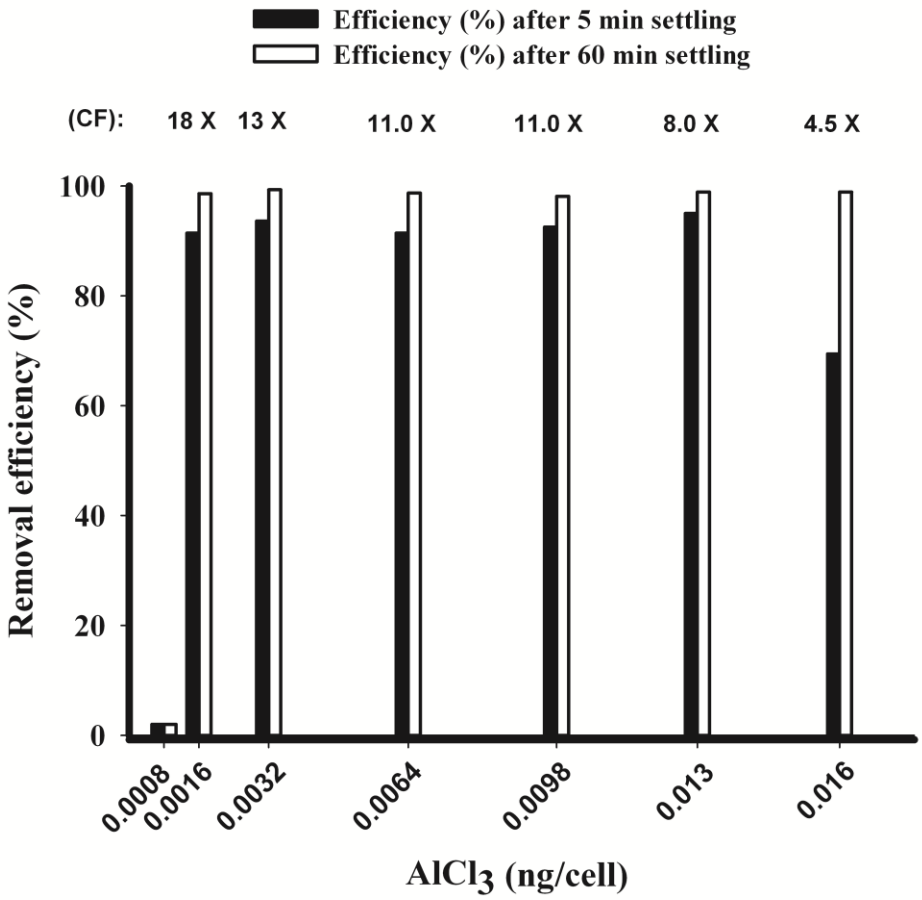

Figure 2.2. Screening of coagulant dosage effect on algae removal efficiency after 5 min and 60 min settling at pH 5.3.

2.4.2 Charge neutralization is not required to achieve > 95 % recovery efficiency

Zeta potential measurements of algae samples at 1.0×10^6 cells/mL, pH 5.0, and $AlCl_3$ concentrations of 0.016 ng $AlCl_3$/cell and 0.0032 ng $AlCl_3$/cell were compared after the rapid mixing (2 min) and slow mixing (15 min) intervals. Figure 2.3 shows complete charge neutralization with 0.016 ng $AlCl_3$/cell, which was achieved after 2 min of mixing. The use of a 5-fold lower coagulant concentration (0.0032 ng $AlCl_3$/cell)

resulted in a small reduction of the zeta potential of about 5 mV. In spite of the substantial difference in the measured zeta potential, the removal efficiency with both dosages (0.016 ng $AlCl_3$/cell and 0.0032 ng $AlCl_3$/cell) was 98.9 % and 99.3 %, respectively. The observed flocculation and algal removal at negative zeta potential of algal slurries agrees with the observation made by Henderson et al. (2008b), who concluded that reduction of zeta potential to approximately -15mV was sufficient to destabilize algal (*M. aeruginosa, C. vularis* and *A. formosa*) slurries and ensure more than 90 % algae removal. For *N. oculata*, reduction of the zeta potential value to about -22 mV was apparently sufficient to achieve destabilization and significant algae removal (> 90 %) (Figure 2.3). Possible reasons for the discrepancy in destabilization zeta potential are the high ionic strength of *N. oculata* media, which contained 15 g/L NaCl, and the 2- to 5-fold greater algae concentration employed in this work compared to that by Henderson et al. (2008b).

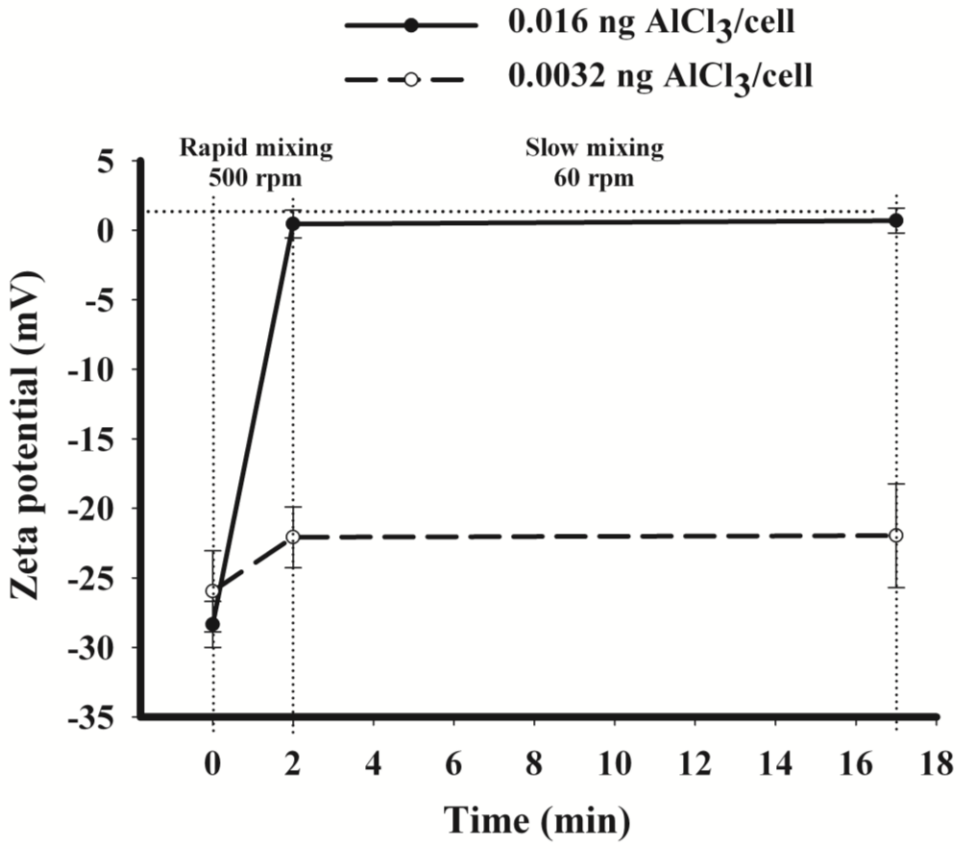

Figure 2.3. Zeta potential measurements at 0.016 ng $AlCl_3$/cell and 0.0032 ng $AlCl_3$/cell concentrations at pH5.0. Bars indicate deviation from the average of two replicates.

2.4.3 Cell concentration and ionic strength (salt) effect on algae removal efficiency

During the screening of flocculation conditions, we noticed that the experiments performed at different cell densities and the same coagulant dosage resulted in slightly different removal efficiencies. Thus, the relationship between initial cell concentration, ionic strength, and required coagulant dosages on removal efficiency was further investigated. Sukenik et al. (1988) showed in previous studies that coagulant dosage required to achieve greater than 90 % microalgae removal increased linearly with water

salinity. To eliminate the effect of EOM on coagulant dosage and to adjust ionic strength of the suspension to a predetermined value, *N. oculata* cells were centrifuged and re-suspended in a salt-free media supplemented with NaCl to the required salt concentration value. Cultures with initial cell concentrations of 4.0×10^6 cells/mL, 3.0×10^7 cells/mL, and 1.0×10^8 cells/mL were flocculated with three coagulant dosages (0.0016 ng $AlCl_3$/cell, 0.0032 ng $AlCl_3$/cell, and 0.01 ng $AlCl_3$/cell) at three NaCl concentrations (0 g/L, 15 g/L, and 30 g/L NaCl). The removal efficiencies as a function of the three variables are summarized in Figure 2.4. A full factorial statistical analysis showed that cell concentration and coagulant dosage had a significant effect on the removal efficiency (P-values < 0.0001 for both factors).

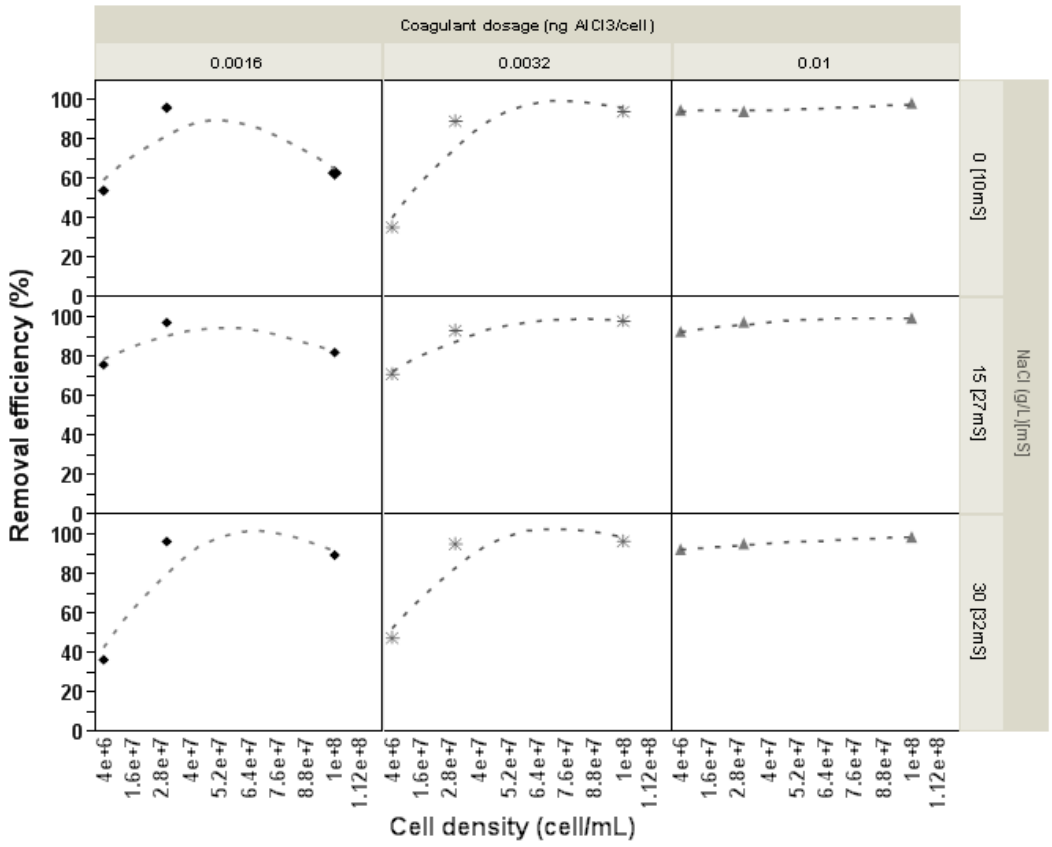

Figure 2.4. Effect of initial cell concentration, coagulant dosage, and NaCl concentration [ionic strength] on harvesting efficiency of *N.oculata* via electrolyte flocculation. Ionic strength is given in brackets on the right ordinate axis. The data reported in this figure is the average of two replicates which deviated less than 10 % for all tested factors (NaCl concentration, coagulant dosage, and cell concentration).

At an initial cell concentration of 4.0×10^6 cells/mL, 0.01 ng $AlCl_3$/cell was required to achieve > 90 % removal efficiency at all three NaCl concentrations. At the other two lower coagulant dosages, 0.0016ng/cell and 0.0032 ng/cell, removal efficiency ranged from 40 % to 60 %.

Although there was a slight difference in the removal efficiency when using an initial cell concentration of 4.0×10^6 cells/mL, the removal efficiencies as a function of the NaCl at each specific coagulant dosage (0.0016ng/cell and0.0032 ng/cell) were not statistically different(p-values < 0.05). When *N. oculata* flocculation was performed with a cell concentration of 3.0×10^7 cells/mL, the removal efficiencies at all three coagulant dosages were at least 90 %. To our surprise, the removal efficiencies of algal suspension with 1.0×10^8 cells/mL and 0.0016 ng $AlCl_3$/cell were 20 % and 10 % lower compared to the 3.0×10^7cells/mL suspensions containing 0 g/L and 15 g/L NaCl, respectively. We expected equal or better removal efficiencies because flocculation kinetics (aggregation rates) is proportional to initial particle concentration (Harrison et al., 2003). The possible explanation for this unexpected discrepancy is the unequal distribution of $AlCl_3$ due to crowing effect in the more concentrated and viscous suspension during the mixing period. Some experiments that we performed on a larger scale using a jar tester and 2 L algae samples with an initial cell concentration of $>10^7$cells/mL did not result in significant flocculation and required a higher stirring speed (40 rpm instead of 20 rpm) during the 15 min slow mixing. After this adjustment, the removal efficiency improved by 25 %. This result suggested that the stirring rate plays an important role on the coagulant distribution, which is reflected in the flocculation efficiency.

As the data in Figure 2.4 show, at least 0.0032 ng $AlCl_3$/cell (100 mg/L) was required to achieve greater than 90 % cell removal when using 10^8 cells/mL algal suspensions. At a dosage concentration of 0.01 ng of $AlCl_3$/cell (300 mg/L), greater than 90 % removal efficiency was achieved at all three initial cell concentrations, indicating that neither the initial cell concentration nor the NaCl concentration affected the removal efficiency when there is excess of coagulant (Figure 2.4).

In summary, several conclusions could be drawn from these experiments. First, initial cell concentration had an effect on the dosage requirement. Second, the coagulant concentration (ng/cell) required to achieve at least 90 % removal efficiency was not proportional to cell concentration. For example, an eight-fold cell concentration (from 4.0×10^6 cells/mL to 3.0×10^7 cells/mL) required (0.01 mg/L/0.0016 ng $AlCl_3$/cell) six-fold lower $AlCl_3$ dosage (ng/cell), whereas from 4.0×10^6 cells/mL to 1.0×10^8 cells/mL a twenty-five-fold cell concentration difference, required (0.01/0.0032 ng $AlCl_3$/cell) three-fold lower $AlCl_3$ dosage (ng/cell). We believe that increase in coagulant dosage with cell concentration measured by Henderson et al.(2008b) and Schlesinger et al. (2012) was due to the presence of EOM, which was removed prior to flocculation in our experiments. Third, the ionic strength effect on recovery efficiency at the dosages and initial cell concentration levels tested was not significant when destabilization was achieved. The effect of ionic strength in this study was more noticeable at a low cell concentration 10^6 cells/mL but was still not significantly different (p-value: 0.9268).

Also, at the highest ionic strength tested (32 mS; 30 g/L NaCl) there was a slight improvement on harvesting efficiency; nevertheless, the ionic strength range from 10 to 32 mS (0 g/L to 30 g/L NaCl) tested in this study did not significantly affect the removal efficiency as it was indicated by the full factorial statistical analysis (p-value: 0.6849).

Flocculation data of *Chlorella vulgaris* (fresh water microalgae) reported by Henderson et al. (2008b) showed that the aluminum ion dosage required to achieve > 90 % removal efficiency at a low cell concentration (5.0×10^5 cells/mL) was 0.0022 ng Al/cell. In this study, we observed that using *N. oculata* at 4.0×10^6 cells/mL required 0.01 ng $AlCl_3$ /cell (0.0020 ng Al/cell) to obtain the same efficiency (Figure 2.5). Furthermore, if the cell concentration of the culture was 3.0×10^7 cells/mL, the coagulant requirement to achieve > 90 % efficiency decreased to 0.00017 ng Al/cell, which is eleven-fold less coagulant (Figure 2.5). This comparison is consistent with our observations that microalgae cultures at lower cell concentrations required greater amounts of $AlCl_3$ for destabilization, and that salinity does not adversely affect the removal efficiency when using aluminum-based coagulants.

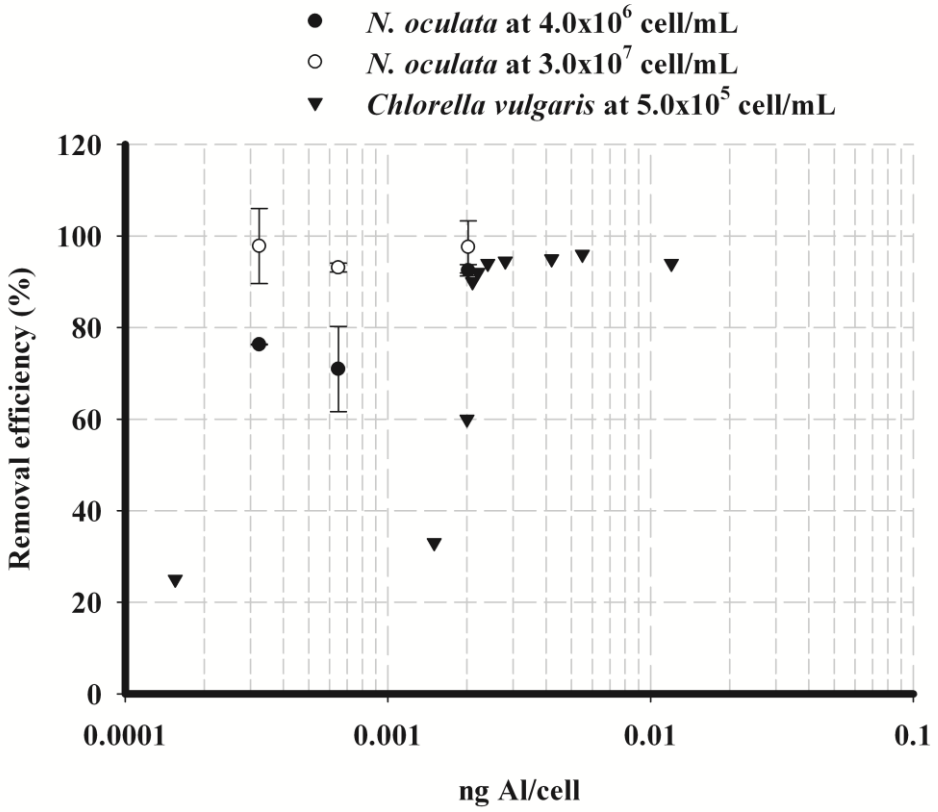

Figure 2.5. Comparison of coagulant requirements for flocculation under optimal conditions of the fresh water species, *C. vulgaris,* and the saline strain, *N. oculata*. Data for *C. vulgaris* was taken from Henderson et al. (2008c).

A positive effect of electrolytes, such as NaCl, on flocculation efficiency should be expected because they are widely used in colloid destabilization to compress the double layer of charged particles (Letterman, 1999) and enhance flocculation rates by increasing the collision frequency factor (Harrison et al., 2003). A previous study by Myers et al. (1975) measured a significant reduction of *N. oculata* electrophoretic mobility (a measure of the zeta potential) with an increase of media salinity (Myers et al., 1975). In this work, the effect of NaCl on flocculation efficiency was studied in the saline

concentration range from 0 g/L to 30 g/L. It was found a rather low impact which could be explained as a masking effect by the hydrolyzed multivalent aluminum ions and the formation of insoluble aluminum hydroxide (sweep flocculation).

We believe that the salinity effect on aluminum sulfate flocculation observed by Sukenik et al. (1988) was caused by the dilution of marine culture with fresh media, which resulted in reduced salinity and also in a decrease of EOM and/or NOM. Therefore, culture dilution reduced the dosage requirement primarily because of reduced EOMs and not due to lower salinity. To estimate the effect of EOM on coagulant dosage, we performed experiments using another marine microalgal culture (*N. salina*) with and without washing the cells (data not shown). We determined that non-washed algal cultures containing EOM required as much as two-fold greater $AlCl_3$ dosage compared with the case when EOM were removed by cell washing.

2.4.4 Optimal removal efficiency variable and apparent sedimentation rate

To find the optimal combination of coagulant dosage and initial cell concentration to achieve maximum removal efficiency, optimization of removal efficiency was performed using Design Expert software (Stat-Ease, Inc.). The two highest efficiencies were obtained at: i) 1.0×10^8 cells/mL and 0.01 ng $AlCl_3$/cell and ii) 3.0×10^7 cells/mL and 0.0016 ng$AlCl_3$/cell (Table 2.1). The lowest removal efficiency (93%) resulted, as expected, using 4.0×10^6cells/mL cultures. With regard to coagulant dosage, 3.0×10^7 cells/mL suspensions would be most desirable because it required 3-fold and 6-fold less coagulant than 1.0×10^8 cells/mL 4.0×10^6 cells/mL, respectively, to obtain 97 % removal.

Table 2.1. Maximization of removal efficiency variable using an optimization function and desirability factors of the Design Expert software.

Initial cell conc. (cell/mL)	Coagulant dosage (ng $AlCl_3$/cell)	Harvesting efficiency (%)	Desirability
1.0×10^8	0.010	98.9	0.990
3.0×10^7	0.0016	96.9	0.964
1.0×10^8	0.0032	96.5	0.959
3.0×10^7	0.010	95.8	0.950
4.0×10^6	0.010	93.2	0.919

Another measure of flocculation efficiency important for process design is the sedimentation rate. Apparent sedimentation rates using optimal coagulant dosage for each initial cell concentration were measured during a 5-min settling period and reported in Table 2.2. As indicated in this table, sedimentation rates depended on the initial cell concentration and coagulant dosage, but were not affected by the salt concentration (ionic strength). The sedimentation rate of 9 cm/h measured with low-concentration algal cultures (4.0×10^6 cells/mL) is too low for effective flocculation on a large scale. Sedimentation rates above 100 cm/h achieved with 3.0×10^7 cells/mL and 1.0×10^8 cells/mL cultures would be adequate for industrial scale microalgae harvesting by flocculation (Stephenson et al., 2010). The highest sedimentation rates were reached using *N. oculata* culture at 3.0×10^7 cells/mL and a coagulant dosage of 0.0016 ng $AlCl_3$/cell.

Table 2.2. Apparent sedimentation rate under optimal coagulant dosage at different cell concentrations.

$4.0x10^6$ cells/mL			**$3.0x10^7$ cells/mL**			**$1.0x10^8$ cells/mL**		
NaCl (g/L)	**ng-$AlCl_3$/cell**	**Sedimentation rate (cm/h)**	**NaCl (g/L)**	**ng-$AlCl_3$/cell**	**Sedimentation rate (cm/h)**	**NaCl (g/L)**	**ng-$AlCl_3$/cell**	**Sedimentation rate (cm/h)**
0	0.01	9	0	0.0016	120	0	0.0032	99
15	0.01	6	15	0.0016	120	15	0.0032	96
30	0.01	6	30	0.0016	126	30	0.0032	102

Significantly lower sedimentation rates measured with 10^6 cells/mL cultures, compared to 10^7 and 10^8 cells/mL, were probably caused by 3 to 6 times greater amount of $AlCl_3$ used for their flocculation, which resulted in flocs bridging and hindered settling (Smith and Davis, 2012).

2.4.5 Coagulant and acid cost

Because acidification of *N. oculata* cultures was required for optimal flocculation (low $AlCl_3$ dosage and greater than 95 % removal), we estimated and compared acidification and coagulation cost. Chemical cost estimate was based on flocculating ~2 g/L *N. oculata* culture (3.0×10^7cells/mL) containing EOM at pH 5.3. Assuming a bulk HCl cost (37.5 % HCl) of $84/metric ton, acidification of *N. oculata* from pH 9 to 5.3 would cost about $0.82/kg of dry weight biomass ($820/ ton dry biomass). $AlCl_3$ at 100 mg/L dosage for unwashed *N. oculata* cultures would cost about $0.054/kg dry weight ($5.4/ton dry biomass) assuming a coagulant bulk cost of $110/kg $AlCl_3$. Clearly, *N. oculata* harvesting cost is determined by the cost of acidification. This is a rather extreme case because of the high-buffering-capacity of *N. oculata* media (15 g/L $NaHCO_3$), which required almost 17 g of 37 % HCl per one liter culture to reduce the pH to 5.3. Selecting a low-buffering-capacity media (with no $NaHCO_3$) for *N. oculata*, such as that used by Wu et al.(2012), should reduce acidification cost at least 15-fold. We have confirmed this hypothesis by flocculating another marine algal culture (*Nannochloropsis salina*) at pH 5.3 using 80 g/L $AlCl_3$. The amount of HCl required to decrease the pH from 9.0 to 5.3 of a *Nannochloropsis salina* culture in a low buffering

capacity media (F/2) was 0.8 g/L HCl, which was 21-fold less than for the high-buffering capacity media. This reduced the HCl cost from $0.82/kg to$ 0.04/kg of dry weight biomass ($40/ton dry biomass). The cost of lime required to raise the pH of a *C. vulgaris* culture for optimal Mg-induced flocculation was recently estimated by Vandamme et al. (2012) at $18/ton biomass. These estimates suggest that the cost of reagents needed to adjust the culture pH in either direction (high or low) for optimal flocculation conditions should not be overlooked.

2.5 Summary

Cell concentration and coagulant dosage had a significant effect on harvesting efficiency via electrolyte flocculation. Coagulant dosage required to achieve significant removal efficiency was not proportional to cell concentration. Best flocculation conditions were achieved at 3.0×10^7 cells/mL and flocculant dosage of 0.0016 ng $AlCl_3$/cell. Ionic strength (sodium chloride concentration) was not detrimental to *N. oculata* flocculation. Flocculation was induced at pH 5.3 and zeta potential of -20 mV indicating that "sweep flocculation" plays a significant role in the suspension destabilization. Acidification algal cultures could be a significant cost burden and can be mitigated by selecting a low-buffering-capacity media.

CHAPTER III

EFFECT OF ALGOGENIC ORGANIC MATTER (AOM) AND SODIUM CHLORIDE ON *Nannochloropsis salina* FLOCCULATION EFFICIENCY*

3.1 Overview

This study evaluates the effect of polymer molecular weight and charge density, algogenic organic matter (AOM), and salt concentration on harvesting efficiency of marine microalgae. Aluminum chloride ($AlCl_3$), chitosan, and five synthetic cationic polymers of different molecular weights and charge density levels were used as flocculation agents. Polymer flocculation of marine microalgae was most efficient when using the highest charge density polymer (FO4990). The flocculant dosage irrespectively of the agent chemistry and charge density was affected by the amount of AOM secreted into the culture media. The presence of AOM increased the amount of required flocculant 7-fold when using synthetic cationic polymers; 10-fold with chitosan; and ~3-fold with $AlCl_3$. Salt concentration of 5 or 35 g/L NaCl alone did not significantly affect removal efficiency, indicating that AOM were the main cause for the increased flocculant dosage requirement. The synthetic cationic polymer (FO4990) was the least expensive flocculation agent.

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*Reprinted from Andrea J. Garzon-Sanabria, Silvia S. Ramirez-Caballero, Francesca E.P. Moss, Zivko L. Nikolov, Effect of algogenic organic matter (AOM) and sodium chloride on *Nannochloropsis salina* flocculation efficiency, Bioresource Technology, 143, 231-237, Copyright 2013, with permission from Elsevier.

3.2 Introduction

3.2.1 Marine microalgae flocculation

Harvesting and dewatering of algal biomass is one of the cost limiting steps in the recovery of algal oil. Several different technologies, including inorganic electrolytes and positively (cationic) charged polymers, have been successfully applied for harvesting fresh-water microalgae by flocculation; however, their effectiveness with marine microalgae are believed to be limited by the presence of salt in the growth media. Studies on commercial harvesting of marine algae biomass by flocculation were reported by Bilanovic et al. (1988) and Sukenik et al.(1988). Sukenik et al. (1988) determined that the dosage of aluminum sulfate and ferric chloride required for flocculation was significantly affected (5-10 greater dosage required) by media salinity and observed a linear increase of flocculant dosage with salinity. The same study also concluded that the flocculation efficiency of marine algae with synthetic (polyacrylamide) and natural (chitosan) cationic polymers have been severely hampered by the ionic strength (salinity) of the media. Flocculation experiments at different ionic strengths were conducted with late exponential phase cultures that were diluted with fresh media to achieve predetermined ionic strengths while maintaining a constant cell concentration of 10^6 cells/mL (Sukenik et al.,1988). Bilanovic et al. (1988) re-examined the effect of sodium chloride (NaCl) concentration on flocculation efficiency using cationic polymers by re-suspending algae in fresh media with different sodium chloride concentrations rather than diluting the algal cultures. Different effects of ionic strength on flocculation efficiencies with two synthetic polymers and chitosan were explained by the difference

in polymer size, molecular configuration, and charge density. Chitosan was the least affected by ionic strength; thus, flocculation of the marine microalgae tested by Bilanovic et al. (1988), required 2-3 fold less chitosan dosage when compared to the amount required by synthetic cationic polymers. The data obtained by diluting the marine cultures with fresh media (Sukenik et al.,1988) could not possibly differentiate the effect of ionic strength and AOM on dosage demand because both salt and AOM concentrations were proportionally reduced by dilution.

3.2.2 Flocculation of fresh water microalgae in the presence of AOM

Although ionic strength undoubtedly affects cationic polymer interactions with negatively charged cells, the potential contribution of AOM (Henderson et al., 2008b; Her et al., 2004; Tirado-Miranda et al., 2003) to the reduction of flocculation efficiency has not been explicitly addressed and/or quantified. Contrary to Sukenik et al. (1988) findings, our recent work demonstrated that $AlCl_3$ flocculation of marine algae (*Nannochloris oculata)* in AOM-free media was not significantly affected by sea salt concentration (Garzon-Sanabria et al., 2012). The growing interest in cost-effective harvesting methods and recycling of spent media spurred interest in AOM released by fresh and marine algae and their impact on harvesting efficiency (Henderson et al., 2008b).

Henderson et al. (2008a) studied the composition of AOM from cyanobacteria (*Microcystis aeruginosa*), green microalgae (C*hlorella vulgaris*), and diatoms (*Asterionella formosa* and *Melosira*). They determined that AOM in the studied algae

species consisted of hydrophilic polysaccharides and hydrophobic proteins whose ratio and concentration differed between the species and with growth phase (Henderson et al., 2008b). Dosage demand for removal of AOM from fresh water microalgae via flocculation with inorganic salts ($FeCl_3$, Alum), decreased as the MW of the proteins and AOM charge density increased (Henderson et al., 2010). The pH of the water also played an important role since excreted proteins and polysaccharides can work as polymer aids (Henderson et al., 2010). Subsequent work determined that the presence of AOM in *Chlorella vulgaris* cultures (at exponential phase) required 1.7-fold more aluminum sulfate for charge neutralization while for *Asterionella formosa* and *Microcystis aeruginosa* required 1.9 and 2.2-fold more aluminum sulfate, respectively (Henderson et al., 2010). These differences were explained by differences in cells charge density, cell morphology, and AOM properties.

Recent studies of Wu et al. (2012) and Vandamme et al. (2012) have also showed that AOM could significantly impact flocculation efficiency. The pH-induced flocculation of *Chlorella vulgaris* at pH 10 was significantly affected (only 50 % removal efficiency) by the presence of 25 mg/L polysaccharides in the media (Wu et al., 2012), but the same pH and carbohydrates concentration only slightly (10 %) reduced flocculation of *N.oculata* (marine) culture. Vandamme et al. (2012) compared the effect of AOM on flocculation efficiency of *C. vulgaris* using five different flocculation methods (alum, chitosan, pH-induced flocculation, cationic biopolymers, and electrocoagulation). To achieve 85 % of algal biomass removal, pH-induced flocculation

required a pH increase from pH 10.5 to 11.5, a 9-fold greater dosage of chitosan (from 8 mg/mL to 75 mg/mL), and about 6-fold greater concentration of cationic starch or alum.

3.2.3 Polymer flocculation of marine microalgae

Since the studies by Sukenik et al. (1988) and Bilanovic et al. (1988), there have been relatively little additional published data on marine algae flocculation with synthetic polymers. Natural chitosan polymer has been given more prominent place in scientific community probably because of its environment–friendly properties (Kumirska et al., 2011).

Chitosan flocculation of eleven marine microalgae species was investigated by Lubian (1989); six out of the eleven species required pH pretreatment to enhance flocculation efficiency. Chitosan flocculation was found to be pH sensitive for fresh and marine water species. Optimal pH for fresh water microalgae was ~ pH 7.0 while, for marine microalgae it required initial adjustment to pH 5.0 - 6.5, followed by the addition of chitosan, and then readjustment to pH 7.0-8.0 (Divakaran and Pillai, 2002; Lubián, 1989).

Uduman et al., (2010b) showed > 78 % flocculation efficiency of marine microalgae with ionic and non-ionic polymers in a dosage range of 2-10 mg/L. The highest removal efficiency (> 85%) was obtained with cationic polymers at 3-4 mg/L, ~ 84 % was achieved when using anionic polymers in a range of 2-5 mg/L, and the lowest flocculation efficiency (~ 78 %) was achieved with a non-ionic polymer of 10 mg/L. Considering the body of previous published work of polymer interactions with algae and

bacterial cultures, it is surprising to see that non-ionic and anionic polymers were only 10-15 % less efficient than cationic polymers. Furthermore, the effect of storing biomass for 168 h prior to flocculation is unclear and might explain the discrepancy between the results obtained by Sukenik et al. (1988) and Uduman et al. (2010) with regards to polymer flocculation.

The present study aims to understand polymer flocculation of marine microalgae and the variables affecting its flocculation efficiency. The objectives of this work were: i) to screen flocculation of *N. salina* with synthetic polymers of different molecular weight (MW) and/or charge density, ii) to revisit the previously observed effect of ionic strength on cationic polymer flocculation, and iii) to quantify the effect of AOM on flocculant dosage apart from NaCl (ionic strength effect).

3.3 Materials and methods

3.3.1 Materials

Synthetic cationic polymers used for flocculation experiments (FO and FL series) were obtained from *SNF FLOERGER* Company (France). Chitosan was purchased from Sigma Aldrich Company (USA), and $AlCl_3$ was purchase from Alfa Aesar Company (USA).

3.3.2 Methods

3.3.2.1 Algae growth and culturing conditions

Nannochloropsis salina, green microalgae species, obtained from Texas AgriLife Research Station at Pecos was selected for harvesting studies via flocculation. Initial inoculums (10^5 – 10^6 cells/mL) were used to scale up the cultures to 1.5 L flasks. *N. salina* was grown in a modified F/2 media (Guillard, 1962), enriched 10-fold with nitrogen source (0.75g/L $NaNO_3$) and $Na_2SiO_3 \cdot 9H_2O$ depleted. Cultures were grown at room temperature (25 °C) under continuous illumination of white light at ~100 PPF (μmol/(m^2·s)) until early stationary phase (~10^7cells/mL) was reached. The pH was maintained between 8.0 and 8.8 by adding a mixture of 3.75 % CO_2 in air when needed. Mixing was provided by sparging air sterilized with 0.2 μm filters continuously into the cultures.

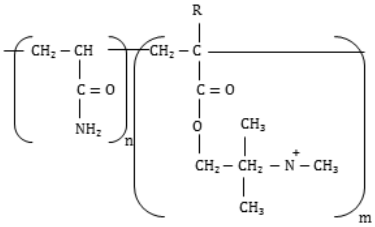
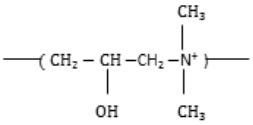
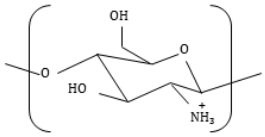
N. salina growth curve was developed to identify different stages of growth for better control of the harvesting time. Two independent cultures of *N. salina* algae strain were inoculated with inoculum of 1.0×10^5 cells/mL and grown for two weeks under the above conditions. Cell concentration was monitored daily by measuring the optical density at 750 nm wavelength using a Beckman Coulter (Brea, CA) DU640 spectrophotometer and cells were counted using a hemocytometer device (BrightLine, Hausser Scientific, Horsham, PA). Total soluble protein and carbohydrates were measured after 1 and 2 weeks of continuous growth. Additionally, culture conductivity was monitored throughout the growth and used as a control parameter between different batches of cultures at specific time points i.e., 1 and 2 weeks of growth.

3.3.2.2 Flocculation experiments

Flocculation agents were prepared in concentrated stock solutions and then added to the cultures at different concentrations to determine the minimum amount required to achieve more than 90 % biomass removal efficiency. Stock solutions were prepared as follow: i) $AlCl_3$ at 40 g/L in water, ii) chitosan stock solution at 10 g/L in 1 % acetic acid solution and mixed for 24 h at 60 rpm, iii) FO- and FL-series polymer stock solutions at 1 and 10 g/L in water, respectively, and mixed for 1 h at 600 rpm. All stock solutions were freshly prepared prior to each flocculation experiment.

Aliquots of 50 mL algae culture were tested in 100 mL glass beakers using seven different flocculants. See flocculant properties in Table 3.1. Mixing was provided with stir bars on a magnetic stir plate and the mixing time was adjusted based on the flocculation agent. All flocculants except for FO4990, were rapidly mixed for 2 min at 500 rpm followed by 15 min slow mixing at 60 rpm to promote aggregation. Mixing conditions when using FO4990 polymer were 40 s rapid mixing at 500 rpm followed by 5 min slow mixing at 200 rpm.

Table 3.1. Physicochemical properties of flocculants used in this study

Flocculant	Molecular weight range ($\times 10^6$ Da)	Active charge density (mole %)	Macro structure	Chemical structure
FO4450	4.2-7.8	45 %	Linear	 Polyacrylamide
FO4650	4.2-7.8	55 %	Linear	
FO4800	7.8-10.0	80 %	Linear	
FO4990	4.2-7.8	99 %	Linear	
FL2949	0.01	100%	Branched	 Polyamine
Chitosan	0.31-0.375	nd[1]	Linear	 β-(1-4)-linked D-glucosamine
$AlCl_3$	-	-	-	

[1]nd: not determined

For optimal flocculation of marine microalgae with $AlCl_3$, culture pH was adjusted to 5.3 and kept constant during mixing (Garzon-Sanabria et al., 2012). For optimal flocculation of marine microalgae using chitosan, algae cultures were acidified to pH 6.5 with 1 M HCl, and after the addition of chitosan stock solution adjusted to pH 8.0 with 1 M NaOH. After mixing, 50 mL samples were transferred to a 50 mL gravimetric cylinder. Optical density at 750 nm was periodically measured over a 1 hour period at 5 cm below the top of the gravimetric cylinder to monitor algal settling. Flocculation experiments were performed in duplicates.

3.3.2.3 Algogenic organic matter (AOM) and salt concentration (NaCl) effect on marine microalgae flocculation

Three different sets of flocculation experiments were performed. The first set was designed to determine flocculation efficiency of *N. salina* cultures in the presence of AOM. All cultures were grown to early stationary phase and have the following properties: cell concentration of ~1.0×10^7 cells/mL,OD_{750} ~ 3.0, and conductivity ranging from 41 to 45 mS/cm. The second set of experiments was conducted with the same early stationary phase cells that were centrifuged at 6000 × g for 7 min using a Beckman Coulter Allegra^{TM} 25R centrifuge (USA). After centrifugation, the supernatant was discarded and the cell pellet was resuspended in fresh media. The AOM–free suspensions had cell concentration of 1.0×10^7 cells/mL,OD_{750}~ 3.0, and conductivity of ~ 30mS/cm. Modified F/2 growth media in both sets contained 20 g/L NaCl and the conductivity difference between the two sets of experiments was due to the presence and

absence of AOMs respectively. Conductivity measurements in these experiments were used to control batch-to-batch variation in ionic strength and avoid potential salt concentration effects. The third set of experiments was designed to determine the effect of salt concentration (NaCl) alone, without the presence of AOM. Early stationary-phase cultures were centrifuged and the cell pellets were resuspended in fresh media containing 5 or 35 g/L NaCl.

3.3.2.4 Removal efficiency calculation

Algal biomass removal efficiency after each flocculation experiment was determined using the optical density data measured at 750 nm wavelength. Settling of flocculated biomass took place in a 50 mL gravimetric cylinder and the samples for optical density determination were taken 5 cm below the top of the gravimetric cylinder at time zero and after 1 h of settling at the same reference point. The algal biomass removal efficiency (RE) was calculated using (eq 3.1) where the initial (OD_0 = before adding flocculation agent) and final (OD_f = after 1 h settling) optical density data was replaced.

$$RE = \left[1 - \left(\frac{O.D_f}{O.D_0}\right)\right] * 100 \qquad \text{(eq 3.1)}$$

3.3.2.5 Total carbohydrates and total soluble protein determination

Total carbohydrates expressed as glucose equivalents were determined by the phenol-sulfuric acid method (Dubois et al., 1956). Total soluble protein was quantified

using the micro-microplate protocol (working range of 1-25 ug/mL) Coomassie plus (Bradford) assay kit (Thermo Scientific). Adsorption measurements were taken using the $VERSA_{max.}$ microplate reader (Molecular Devices, CA) at 595 nm wavelength.

3.3.2.6 Calculation of reagent cost for optimal flocculation conditions

Total reagent cost was calculated by taking into account flocculating agent cost and required amounts of acid and/or base to adjust culture pH for optimal flocculation. $AlCl_3$ performed best when cultures were pH adjusted to pH 5.3 with acid. Likewise, the flocculation of *N. salina* with chitosan required a two-step process for pH adjustment to improve the algal biomass removal efficiency. Synthetic cationic polymers did not require pH adjustment. The cost of FO4990 of $4.0/kg and chitosan of $7.0/kg was provided by *SNF FLOERGER* Company. $AlCl_3$ cost was estimated to be ~ $1.10/ kg. To express the reagent cost per kg dry weight of algae, we used previously determined dry weight of 0.7 g/L for 1×10^7 cells/mL *N.salina* culture.

3.4 Results and discussion

3.4.1 Charge density effect of synthetic cationic polymers in marine microalgae flocculation

The preliminary screening of a broad range of commercial (coagulants and flocculants) synthetic polymers from *SNF FLOERGER* Company established that flocculants with a MW greater than 10^6 Da were most efficient in initiating flocculation of *N. salina* cultures. The best performing polymer was FO4990 (Table 3.1), which at 20

- 30 g/L dosage achieved > 90 % removal efficiency while the other three linear polyacrylamide polymers required higher amounts to achieve the same flocculation efficiency. The lower MW branched, polyamine polymer (FL2849) did not result in a substantial level of flocculation when using the same *N. salina* cultures even at concentrations of 100 g/L. Further investigation of *N. salina* flocculation included the five synthetic polymers listed in Table 3.1. When necessary, chitosan and $AlCl_3$ were also included in the analysis for comparative purposes.

The effect of polymer charge density on flocculation efficiency of four FO-series polymers was compared at a constant dosage of 20 mg/L (Figure 3.1). *N. salina* cultures in these experiments contained ~ 1.0×10^7 cells/mL and had a conductivity of 40 mS/cm. The plotted trend in Figure 3.1 indicates that polymers with higher charge densities were more effective in flocculating *N. salina* cells. The highest flocculation efficiency of 93 ± 0.7 % was achieved with the polymer FO4990 and the lowest (73 ± 4.2 %) with FO4550. The biomass removal efficiencies of the other two polymers FO4800 and FO4650 were both about 80 % and not significantly different. The low MW polyamine FL2949, even though it has a 100 % active charge density, did not induce cell flocculation at 20 g/L probably because of its inability to bridge algal cells.

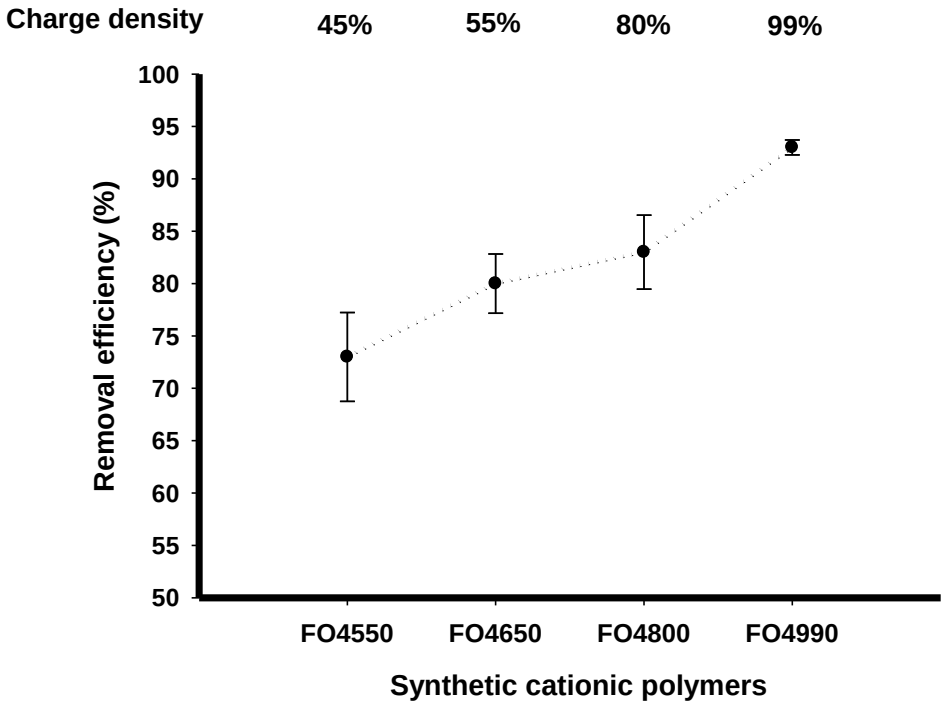


Figure 3.1. Removal efficiency of *N. salina* cells (~ 1.0×10^7 cells/mL) using synthetic cationic polymers at a dosage of 20 g/L. Flocculation experiments performed in the presence of AOM. Bars indicate standard deviation for experiments performed in triplicates.

These results indicate that flocculation of marine microalgae with cationic polymers is affected by active charge density and molecular weight. Therefore, observed correlation between charge density and flocculation efficiency is valid for polymers of similar molecular weight. The data from this study shed light on previous reports of poor flocculation efficiencies of marine microalgae with polyacrylamide cationic polymers, Zetag 63 and Zetag 92, also at 20 mg/L. Since these two polymers have similar MWs (1×10^7 and 2×10^7 Da respectively), the weaker flocculation power of Zetag 92 compared to Zetag 63 was explained by the difference in charge density, implying that Zetag 92

was the polymer with a lower charge density and, thus was affected more by ionic strength (media electrolytes) than Zetag 63. To support the premise that highly-charge polymers are less sensitive to the presence of media electrolytes, we compared the effect of NaCl alone using *N. salina* resuspended in AOM-free media. Flocculation experiments were performed by resuspending the cells in fresh growth media with pre-adjusted NaCl concentrations to 5 g/L and 35 g/L (Figure 3.3-2). The conductivity of resuspended cultures was 8.1±0.14 mS/cm for 5 g/L NaCl and 43.5±0.5 mS/cm for 35 g/L salt-containing media. To achieve greater than 90 % removal efficiency for low-ionic strength suspensions, the dosage of the synthetic polymer FO4990 had to be increased from 3 to 4 mg/L. Therefore, biomass removal efficiencies (%) of all synthetic cationic polymers in Figure 3.2 were compared at 4 mg/L. As data in Figure 3.2 indicates, the difference in salt concentration did not significantly affect individual and relative removal efficiencies of each polymer (i.e. FO4990 was the most efficient at both salt concentrations followed by FO4880, FO4650, and FO4550). Likewise, chitosan which has been found in previous studies to be affected by high ionic strength was not significantly affected by NaCl concentrations of 5 or 35 g/L. Chitosan dosage had to be increased from 5 to 8 mg/mL to flocculate *N. salina* suspensions from low-ionic strength media containing 5g/L NaCl, similar to synthetic polymers. The lower dosage requirement for algal flocculation at 43.5±0.5 mS/cm compared with 8.1±0.14 mS/cm observed in this study is consistent with the compression of the double layer caused by an increase in ionic strength (Harrison et al., 2003).

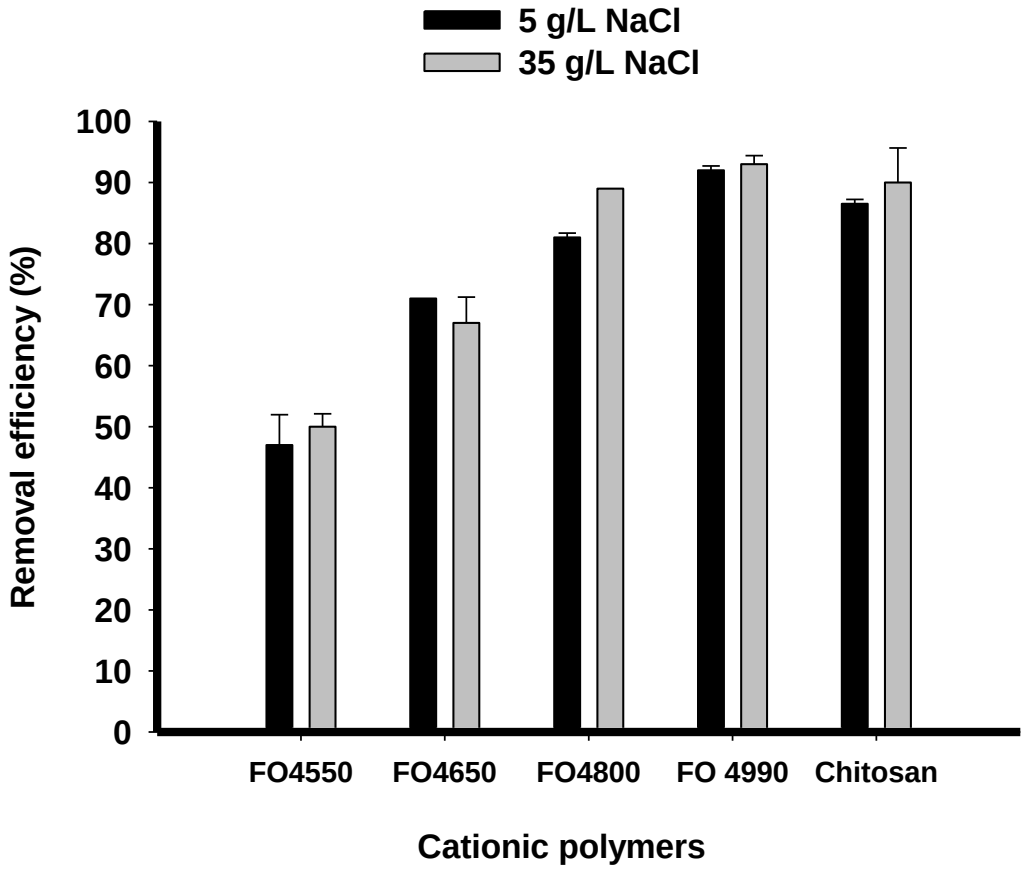


Figure 3.2. Salt effect (NaCl) on *N. salina* removal efficiency when using synthetic cationic polymers at a dosage of 4 mg/L and chitosan at 8 mg/L in media containing 5 g/L and 35 g/L NaCl. Flocculation experiments performed in the absence of AOM. Bars indicate deviation from the average of two samples.

Our results suggest that significant flocculation efficiency of marine microalgae can be achieved at high salt concentrations if highly charged density polymers are used. Salt (NaCl) concentration alone was not an impediment to flocculation and in fact, it slightly improved flocculation efficiency. Our previous work (Garzon-Sanabria et al., 2012) also showed that flocculation of *N.oculata* with aluminum chloride was not affected by NaCl concentration in the media. Therefore, it appears that the extra amount of FO-series

polymers (20 g/L) required to flocculate marine microalgae (Figure 3.1) was due to culture AOM and not the high-ionic strength of the media.

3.4.2 Impact of AOM on marine microalgae flocculation with seven different flocculation agents

The purpose of performing flocculation experiments for *N. salina* with and without the presence of AOM was to compare required flocculant dosages under both conditions. To establish the impact of AOM on algal flocculation, in addition to synthetic polymers, we investigated flocculation with chitosan and $AlCl_3$ (Figure 3.3). The minimum dosages required to achieve > 90 % cell removal using FO 4990 polymer, chitosan $AlCl_3$, and FL2949 were first determined using AOM-free cultures (Figure 3.3a). The linear polyacrylamide polymers were compared at a polymer concentration of 3 mg/L, which was the minimum concentration of FO4990 required to obtain > 90 % removal. The reason for comparing the three FO polymers to FO4990 was to ascertain the previously observed decreasing trend in removal efficiency that paralleled the decrease in charge density of the same polymers in the presence of AOM (Figure 3.1). Results of these experiments show that 7-fold greater concentration of linear synthetic cationic polymers (i.e. FO4550, FO4650, FO4800, and FO4990) was required in the presence of AOM compared to AOM-free cultures. The percentage of removal efficiencies in the absence of AOM (Figure 3.3a) and in the presence of AOM (Figure 3.3b) correlated to charge density of the synthetic polymers; FO4990 being the most effective (> 90 % removal efficiency) and 4550 the least with 70 % removal. A similar (6-fold) reduction

in removal efficiency was also observed by Vandamme et al. (2012) when using cationic starch for flocculation of *Chlorella vulgaris* (freshwater microalgae).

The smaller molecular weight (1×10^4 Da) polyamine-based polymer FL2949 was the least effective among cationic polymers, requiring 100 mg/mL concentration in the absence of AOM to achieve a removal efficiency of 90 % (Figure 3.3a), but the maximum achievable flocculation efficiency in the presence of AOM was only 65 % (Figure 3.3b).We believe that a 100-fold difference in molecular weight compared to the FO-series polymers ($>10^6$ Da) significantly affected the performance of FL2949 polymer. Even though FL2949 has 100 % active charge density, its size probably was not sufficient to effectively bridge *N. salina* cells and form flocs. Therefore, the behavior of this polymer was more coagulant-like and hence, required a higher dosage to destabilize the system by charge neutralization.

$AlCl_3$ was relatively efficient at 50 mg/mL in the absence of AOM and required a 3-fold greater dosage to achieve 90 % removal in the presence of AOM. The 3-fold concentration increased, falls between the 2-fold greater dosage of aluminum sulfate required for charge neutralization of freshwater algae containing AOM (Henderson at al., 2010) and 6-fold to achieve 85 % removal of *Chlorella vulgaris* (Vandamme et al., 2012). Flocculation with chitosan was similarly affected by AOM. Published data show that 10-fold more chitosan was required to flocculate *N. salina* cells (Figure 3.3) and 9-fold more was needed to flocculate *Chlorella vulgaris* (Vandamme et al., 2012).

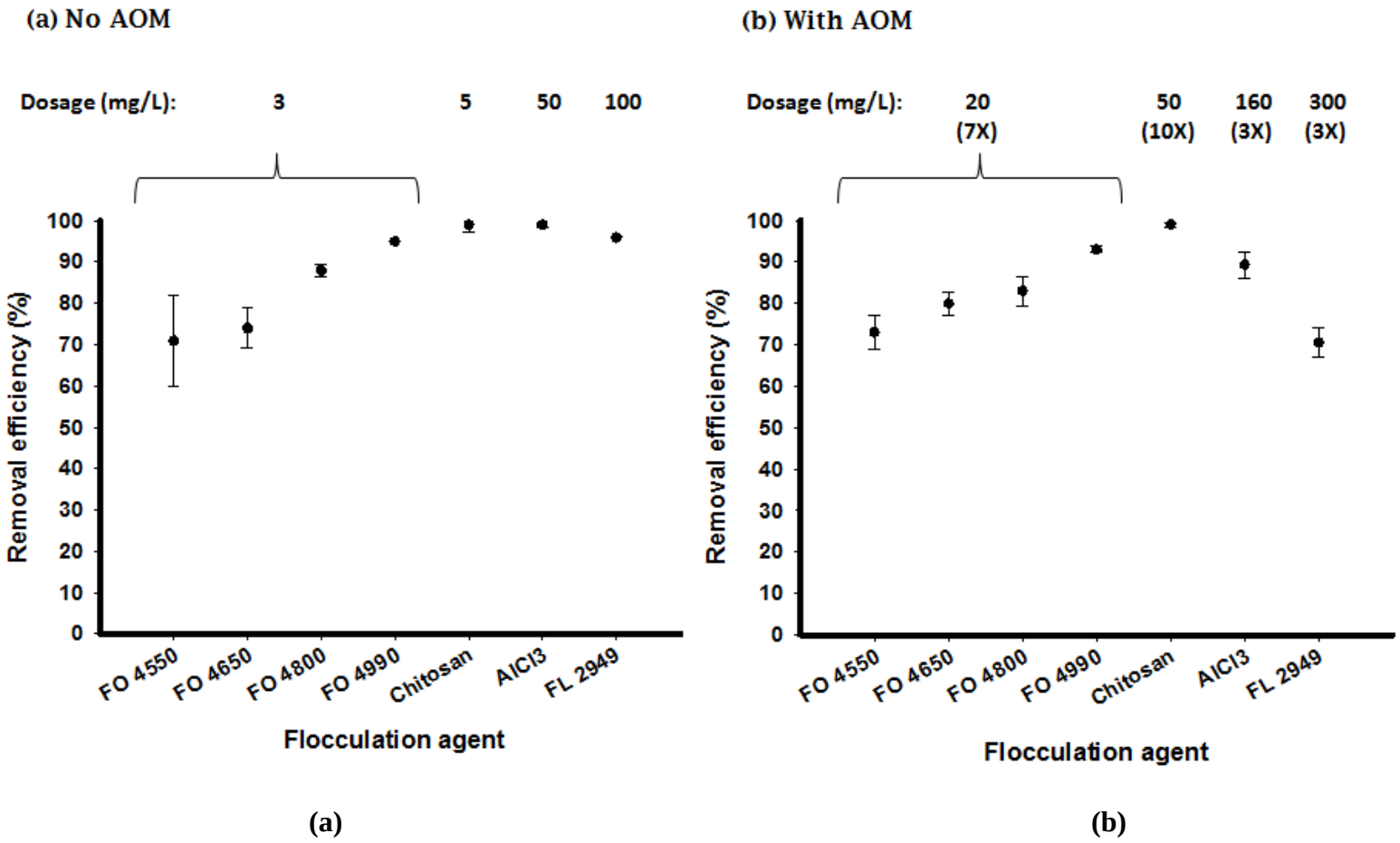


Figure 3.3. AOM effect on flocculation of *N. salina* with seven different flocculation agents. (a) Algal biomass removal efficiency (%) in the absence of AOM present in the culture and (b) Algal biomass removal efficiency (%) in the presence of AOM. Bars indicate standard deviation of experiments performed in triplicates.

Our findings show a greater dosage demand of synthetic cationic polymers, chitosan, and aluminum chloride when AOM is present in marine algae cultures. The data agree with the increased demand of alum, chitosan, and cationic starch required for flocculation of freshwater microalgae reported by Vadamme et al. (2012) and Henderson et al. (2010). Although no absolute correlation is possible, the presence of salt does not appear to be the main cause of increased flocculant demand, but AOM.

3.4.3 Contribution of secreted protein and carbohydrates in flocculant dosage demand for *N. salina* flocculation

Previous studies on flocculation of fresh water microalgae found that AOM, which may contain polysaccharides, proteins, small organic molecules, nucleic acids, and lipids (Henderson et al., 2008b; Pivokonsky et al., 2006), interfered with flocculation mainly due to protein- and/or polysaccharide-flocculant interactions (Henderson et al., 2008b; Henderson et al., 2010). In this work, we estimated total soluble protein (TSP) and carbohydrate concentrations in the media at two points during the growth phase of *N. salina* in an attempt to correlate required flocculant dosage to the amount of protein and/or polysaccharides excreted into the media. The concentration of TSP and carbohydrates secreted to the media during *N. salina* growth was measured during two-weeks of growth. The biggest amount of TSP detected after 1 week of growth was 4.1 ± 0.2 mg/L and 8.8 ± 1.0 mg/L after the second week (Figure 3.4).

Carbohydrate concentration in the growth media was 100 ± 12.1 mg/L (8.4 ±1.0mg C/L) after 1 week and 223.3 ± 18.2 mg/L (18.6 ±1.5 mg C/L) after 2 weeks of growth. The carbohydrate concentration in the early stationary phase media of *N. salina* is similar to the carbohydrate content (5 mg C/L) determined in *Chlorella vulgaris* growth media by Vandamme et al.(2012b). Carbohydrates accumulation of *N. salina* after a week of growth (100 mg/L) was 4-fold greater than the amount of polysaccharides (25 mg/L) released by *Chlorella vulgaris* after the same growth time (Wu et al., 2012). Wu and co-workers (2012) determined that at a polysaccharide concentration of 25 mg/L, flocculation efficiency was reduced by 10 % when using *N. oculata* and by 50 % when using *Chlorella vulgaris.* Based on the low protein concentration in the growth media, we do not believe that the excreted proteins played a major role in the reduction of flocculant efficiencies, but rather, carbohydrates at 100 mg/L are more likely to have contributed to the increased dosage requirements of flocculants reported in Figure 3.3.

	1 week	2 weeks
Total soluble protein (TSP mg/L)	4.0 ± 0.2	8.0 ± 1.4
Total carbohydrates (mg/L)	100.6 ± 12.1	223.3 ± 18.2

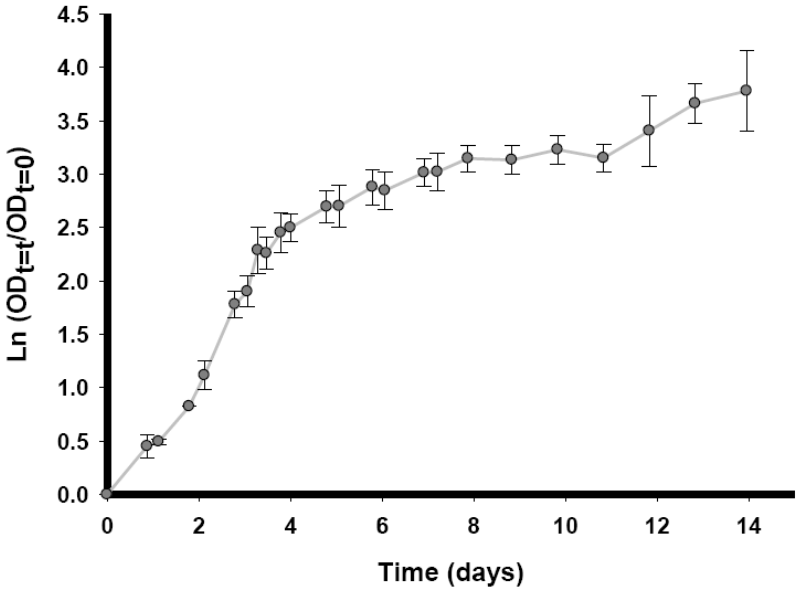


Figure 3.4. *N. salina* growth curve with total carbohydrates and total soluble protein (TSP) concentration after 1 and 2 weeks of growth. The growth curve was constructed based on two independent cultures simultaneously growing and triplicates of each sample were analyzed. Carbohydrate and protein determination were performed in triplicates

To further compare various flocculants, we estimated the total reagent cost required to achieve 90 % removal efficiency of *N. salina* cultures. In addition to polymer cost, the cost of acid used to adjust the pH of *N. salina* cultures for optimal flocculation efficiency was also taken into account. The cost data in Table 3.2 indicates that the synthetic polymer FO4990 was the least expensive flocculant followed by $AlCl_3$ and chitosan. The acidification cost was not a major cost contributor for *N. salina* flocculation when grown

on a small scale in environmentally controlled photobioreactors. The quoted cost of chitosan at $ 7/kg was rather high, and some reports indicate that chitosan production cost can be as low as ~ $2.00/kg (Divakaran and Pillai, 2002).

Table 3.2. Economic analysis for the seven flocculants analyzed in this study

Flocculant	Acid cost ($/kg DW)	Flocculant cost ($/kg DW)	Total cost ($/kg DW)
$AlCl_3$	0.04	0.25	0.29
FO4990	None	0.12	0.12
Chitosan	0.08	0.50	0.58

3.5 Summary

Salt concentration at 5 and 35 g/L alone did not significantly affect *N. salina* removal efficiency, indicating that AOM was the main cause for higher flocculant demand. Results suggest that marine microalgae can be efficiently flocculated by using high-charge density synthetic polymers or chitosan. TSP secreted into the media at concentrations between 4 to 8 mg/L seems rather small to significantly interfere with flocculation of marine microalgae. However, the substantial amount of carbohydrates (100 - 220 mg/L) present in *N. salina* cultures is likely to be the main component of AOM triggering greater flocculant dosage demand for efficient flocculation.

CHAPTER IV

INDUCED CHLOROPLAST EXPRESSION AND RECOVERY OF TWO RECOMBINANT PROTEINS FROM *Chlamydomonas reinhardtii*

4.1 Overview

This chapter addresses cultivation, extraction, and purification of two difficult to produce recombinant proteins. These two recombinant proteins, immunotoxin MT51 and *Plasmodium falcaparum* surface protein25 (Pfs25), are potential products for therapeutic applications. Two significant challenges that limited further process studies with MT51 immunotoxin were: i) low expression level, and ii) significant proteolytic degradation. Although, strain and construct optimization to increase heterologous expression level was performed by our collaborator (Dr. Mayfield), the size and multi domain nature of MT51 made it more vulnerable to proteolytic degradation and no significant improvements could be attained during downstream processing. Our findings suggest that development of protease deficient strains is necessary to determine whether *in vivo* and *in vitro* degradation were the major bottleneck to increase accumulation of the immunotoxin in microalgae chloroplast. The second recombinant protein evaluated was Pfs25 antigen. Maximal biomass accumulation of *C. reinhardtii* expressing Pfs25 was achieved after 5 to 6 days of continuous growth under heterotrophic conditions (no light exposure); however, cell concentration was rather low (~ 10^5 cells/mL). Since no further growth was seen after 6 days, a strategy to increase cell concentration and guaranty nutrient availability for protein synthesis during light exposure was made by

resuspending the biomass in fresh media, incubating ~ 1 day, and then exposing to light for protein synthesis. Using this strategy cell concentration reached 10^6 cell/mL and we guaranty essential nutrients for recombinant protein synthesis. Best conditions of light exposure to produce Pfs25 were 24 h at a photon irradiance of 120 μmol/m^2s. Significant variation was observed among the transformants tested during the screening procedure to select the one that produced higher recombinant protein, which indicates that screening an extensive number of transformants is required. Recovery and purification of Pfs25 was achieved by using anti-FLAG affinity resin; however it is an analytical method that is extremely expensive and not scalable.

4.2 Introduction

4.2.1 *Chlamydomonas reinhardtii* as a model system for complex recombinant protein expression

C. reinhardtii is a unicellular eukaryotic green microalgae that is being used as a model system to express different recombinant proteins of industrial and pharmaceutical interest, because of its good standing genetic characteristics and ease of transformation (Manuell et al., 2007; Mayfield and Schultz, 2004). *C. reinhardtii* can accumulate transgenic proteins in three different ways: 1) expression of the transgene in the nucleus and targeting the protein to the cytoplasm, 2) expression of the transgene in the nucleus and secretion of the recombinant protein via endoplasmatic reticulum and Golgi apparatus, and 3) expression of the transgene and accumulation of recombinant protein within the chloroplast (Griesbeck et al., 2006).

Optimal gene expression in the algal nucleus is still being developed and optimized (Eichler-Stahlberg et al., 2009). Silencing of transgenes via transcriptional and posttranscriptional events is a big disadvantage of randomly inserted transgenes into the nuclear genome (Griesbeck et al., 2006). Chloroplast expression is currently a preferred method for recombinant proteins that do not require posttranslational modification for activity. Some of the advantages of chloroplast expression are: 1) well known tools for chloroplast transformation and improved transgene expression, 2) protected environment from cytoplasmic proteases, and 3) the presence of endogenous disulfide isomerases and chaperone proteins that enable correct heterologous protein folding (Gregory et al., 2012; Mayfield et al., 2003; Tran et al., 2009).

Protein production in the chloroplast of *C. reinhardtii* is a relatively recent achievement (Mayfield and Franklin, 2005; Rasala et al., 2010). Initial attempts to express recombinant proteins in the chloroplast of *C. reinhardtii* under the control of promoters such as: *rbcL, psbA* or *atpA* resulted in none or very low protein accumulation (Blowers et al., 1989; Blowers et al., 1990; Ishikura et al., 1999). Further studies demonstrated that to achieve successful recombinant protein accumulation in *C. reinhardtii* chloroplast expression vectors required codon optimization and insertion of 5' and 3' untranslated regions (UTR) of *atpA*, *rbcL* or psbA genes (Mayfield et al., 2003; Mayfield et al., 2007; Mayfield and Schultz, 2004; Tran et al., 2009). Manuell et al. (2007) also found that the expression of bovine mammary-associated serum amyloid (M-SAA) could be improved 20-fold by replacing the chloroplast *psbA* gene with *psbA-m-saa* gene. In 2010, seven proteins for human therapeutic treatment were selected from a

wide range and were expressed in the chloroplast genome to show their versatility as a platform to produce recombinant proteins; more than 50 % of the proteins were produced at commercial levels (Rasala et al., 2010).

Main factors affecting expression of recombinant proteins in the chloroplast of *C. reinhardtii* identified by Surzycki et al.(2009) were: codon optimization, proteolytic activity, protein toxicity, and random genotypic modifications during transformation. For example, as much as 24 fold difference in recombinant protein levels has been observed for the same transgenes regulated by *psbA* or *atpA* promoter. This difference was attributed to random mutations in the genotypic background during transformation. Transgene expression regulated by *psbA* promoter was significantly higher when compared to transgene expression driven by *atpA*. However, the fact that there was a significant variation between transformants, it made it difficult to conclude which promoter was better for transgene expression (Surzycki et al., 2009). Surzycki et al. (2009) suggested that transformation-associated genotypic modifications in their study was likely to be the main reason for poor protein expression and not gene incompatibility, genetic elements, and insertion sites. To overcome this barrier the authors recommend extensive screening of transformants to isolate the most productive ones for further process development. In cases of reduced yield due to heterologous protein degradation, one could adapt the methods and strategies used to increase stability of proteins expressed in transgenic plants (Potvin and Zhang, 2010). For recombinant proteins that are toxic to the cell, an inducible gene expression provides a useful tool for overcoming that yield constrain.

Rasala et al. (2010) and Mayfield and Franklin (2005) reported an average accumulation level of plastid-expressed proteins in *C. reinhardtii* between 1 % and 5 % TSP. Coragliotti et al. (2011) also discuss factors that can influence expression level of recombinant proteins such as, gene copy number, heterologous gene transcription rate, message stability, and translation of recombinant mRNA. *In vitro* (toeprint assay) and *in vivo* (polysome) analysis revealed that poor ribosome association and translation elongation could have a significant impact on the efficiency of protein expression. According to the results, translation appears to be the rate limiting step in recombinant protein accumulation in *C. reinhardtii* chloroplast (Coragliotti et al., 2011).

Two important classes of therapeutic protein molecules that have been produced in the chloroplast of *C. reinhardtii* include full-length monoclonal antibodies (mAbs) and vaccine antigens. Monoclonal antibodies are complex molecules that have been used for treatment of infectious diseases for more than a century (Tran et al., 2009). These molecules are formed by two heavy chains and two light chains assemble together by 16 disulfide bonds in the endoplasmatic reticulum (Tran et al., 2009). The complexity of these molecules has limited their expression to mammalian cells. A full-length human monoclonal antibody against anthrax toxin has been successfully produced in the chloroplast of *C. reinhardtii,* demonstrating the chloroplast ability to assemble complex molecular proteins (Tran et al., 2009). Because the chloroplast of *C. reinhardtii* lacks the machinery to glycosylate proteins, monoclonal antibodies produced in this organelle are non-glycosylated that lack the ability to bind to Fc receptors and compliment components, which affect the ability of antibodies to recruit killer cells; even though,

recruiting killer cells or fixing complements are key features of antibodies function, there are several therapeutic circumstances were they are not required i.e., for the toxin blocking agent of 83K7C antibody (Tran et al., 2009). Additionally, glycosylation seems to have low impact on antigen binding activity of antibodies (Tran et al., 2009). Antibody-drug conjugates (ADC) are antibody-drug fusions (also referred to as immunotoxins) molecules of higher complexity that have been in development for the past thirty years. ADCs consist of antibody and toxin molecules produced separately and then chemically linked (Kreitman, 2009). The toxin part is commonly produced in bacteria or chemically synthesized, while the antibody is expressed and purified from mammalian cells. The entire process of synthesizing ADC parts in different expression systems followed by purification, conjugation, and re-purification of the conjugate is low-yielding and costly process. More importantly, multiple manipulation steps affect molecule stability that may result in the release of the toxin and cause nonspecific cytotoxicity (Kreitman, 2009). Several immunotoxins have successfully been tested in clinical trials (Choudhary et al., 2011; Kreitman, 2009; Lechleider and Pastan, 2011), but only Denileukin diftitox and Brentuximab vedotin are currently FDA approved ADCs. Denileukin diftitox is a fusion protein of truncated diptheria toxin (DT) and interleukin 2 (IL2) used for patience with refractory cutaneous T-cell lymphomas. Brentuximab vedotin, approved by FDA in 2012, targets CD30 antigen expressed by activated B and T cells and on both Hodking's lymphoma (HL) and systemic anaplastic large cell lymphoma (ALCL). The construct of Brentuximab vedotin comprises a monoclonal antibody (cAC10) against CD30, the microtubule disrupting agent monomethylauristatin

E (MMAE), and a protease-cleavage that holds together cAC10 to MMAE. Being able to express ADC molecules in algal chloroplast provides an opportunity to decrease manipulation steps during manufacturing of immunotoxins and consequently reduction of production cost.

Production of recombinant molecular vaccines is another important opportunity for transgenic microalgae and plant biotechnology, especially when attenuated vaccines are difficult to produce due to safety concerns (Bruder et al., 2010). Malaria represents a classic example of diseases that provides opportunities for developing subunit vaccines because of multiple parasite-host molecular interfaces. The second generation malaria vaccines targeting asexual blood and sexual stages of the parasite are currently in development (Anders et al., 2010; Arévalo-Herrera et al., 2011). Vaccines targeting asexual blood are aimed to stop invasion of the parasite, whereas, those targeting sexual stages of the parasite aim to prevent parasite fertilization and/or development in the mosquito vector (Clemente and Corigliano, 2012). Transmission-blocking vaccine (TBV) is considered a potential solution for malaria. This type of vaccine uses an antigen that elicits antibodies that recognize specific protein on the gametocytes or parasite development stages and inhibit reproduction of the parasite (*Plasmodium falcaparum*) in the midgut of the anopheles mosquito (Clemente and Corigliano, 2012). *Plasmodium falciparum* surface protein 25 (Pfs25) and 28 (Pfs28) are outer membrane proteins that has been identified to play an important role in the sexual stages of development of the *plasmodium* parasite by blocking their maturation process (Gregory et al., 2012). These proteins, which are nonglycosylated and structurally complex

moleculeswith several disulfide bonds, have been difficult to produce in *Escherichia coli* because of incorrect structure of the protein (Kaslow et al., 1992), in yeast due to multiple conformations (Gozar et al., 1998; Kaslow and Shiloach, 1994), and in tobacco plants due to post-translational glycosylation (Farrance et al., 2011). Gregory et al. (2012) succesfully expressed Pfs25 antigen in the chloroplast of microalgae, demonstrating that microalgae chloroplast is the first recombinant system to produce an unmodified version of Pfs25 antigen.

4.2.2 Immunotoxin (CD22-HCH23-ETA) construct

The most recent work by Mayfield lab demonstrated that expression of immunotoxin in *C. reinhardtii* chloroplast was possible (Tran et al., 2012). An immunotoxin molecule, CD22-HCH23-ETA, (herein, referred as MT51) has been constructed by fusing a truncated variant of *Pseudomonas aeruginosa* exotoxin A (ETA) to an antibody fragment that recognizes the CD22 antigen on B-cells leukemia and lymphomas (Lechleider and Pastan, 2011). In the MT51 constructs, domain Ia of the native exotoxin A has been removed in order to avoid binding of the toxin to normal cells, and the last five amino acids (REDLK) of the C-terminal domain III were replaced with the KDEL sequence. The KDEL sequence is recognized by the intracellular sorting receptors that direct the carboxyl terminal fragment to the endoplasmic reticulum. MT51 molecule does not contain the heavy (CH1) and light (C_L) constant regions of the Fab part of the antibody but it contains the light chain variable region (scFv) linked to the six-amino-acid FLAG-tag (Asp-Tyr-Lys-Asp-His-Asp). Binding domains of the antibody against

CD22 were cloned into a single chain fragment (scFv) and fused with ETA coding region. The hinge and constant domains 2 and 3 (HCH23) were added in between the scFv and ETA regions as shown in Figure 1. Endogenous *psbA* gene coding for D1 protein, was replaced by the anti-CD22scFv-HCH23-ETA immunotoxin (MT51) via direct homologous recombination of the *psbA* locus, by using a chloroplast expression cassette that contained immunotoxin and kanamycin (kan) resistance coding region. Since recombinant protein expression is controlled by the *psbA* promoter, transgene expression is light inducible, which provides the advantage of controlling biomass accumulation and transgene expression separately. *C. reinhardtii* expressing recombinant MT51 is a non-photosynthetic algae strain that has the ability to grow under heterotrophic conditions (in the dark). Expressed MT51 conjugate assembles as a dimer through disulfide bonds formed in the hinge region of the molecule and has an estimated molecular weight (MW) of 190 kDa and isoelectric point (pI) of 5.5. The immune affinity tag (FLAG) attached to N-terminus of the molecule is used for detection and purification of MT51 from clarified algal homogenate by anti-FLAG affinity chromatography.

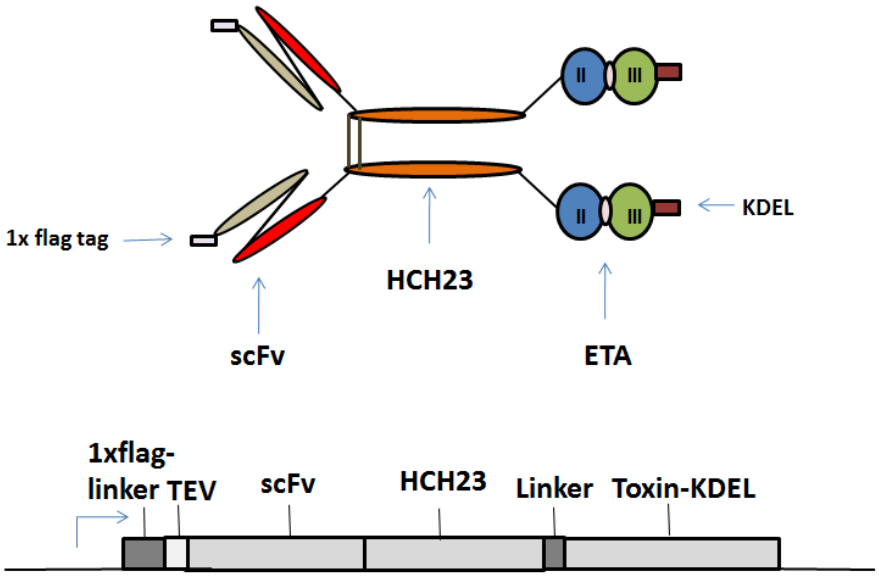


Figure 4.1. Immunotoxin MT51 depiction and expression cassette. The scFv stands for single chain antibody and ETA for exotoxin A. (Courtesy of Dr. Mayfield).

4.2.3 Pfs25 antigen construct

Pfs25 antigen is a complex protein that contains four epidermal growth factors-like domains (EGF), each with several disulfide bonds. *C. reinhardtii* chloroplast was transformed using the expression cassette depicted in Figure 4.2. This construct contains a codon optimized nucleotide sequence corresponding to the EGF domains 1 to 4 of Pfs25, which were cloned into an open reading frame upstream of a TEV protease site and FLAG epitope. Similarly to MT51 strain, endogenous *psbA* gene coding for D1 protein, was replaced by *Pfs25* via direct homologous recombination at the *psbA* locus. Transgene expression is regulated by the *psbA* promoter and the 5' and 3' untranslated regions (UTRs) and, therefore, light inducible.

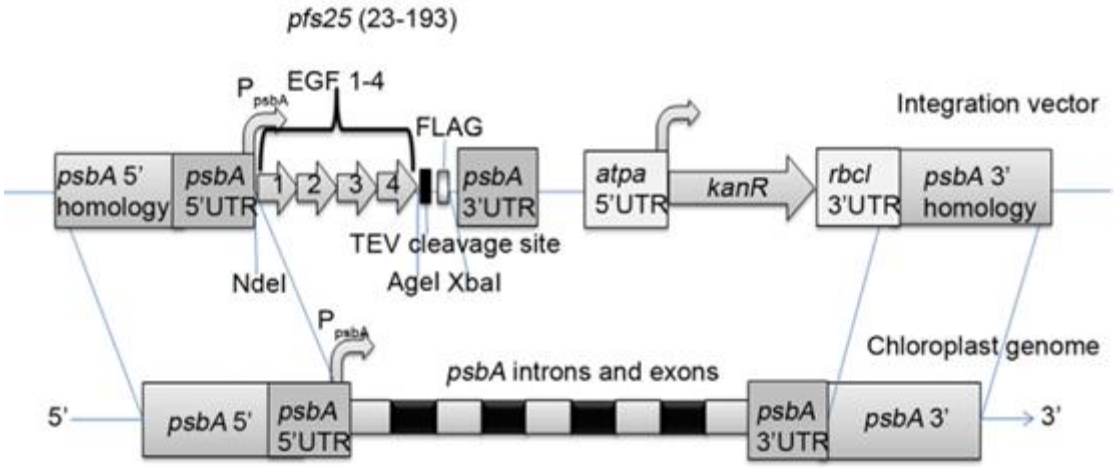


Figure 4.2. Diagram of chloroplast transformation vector to express recombinant Pfs25 antigen. Taken from Gregory et al. (2012)

The purpose of this study is to optimize *C. reinhardtii* biomass production and induction time (light exposure) to maximize accumulation of recombinant proteins expressed in the chloroplast of *C. reinhardtii*. Although production of complex recombinant proteins in microalgae chloroplast has been demonstrated, current protein isolation and purification method using FLAG affinity interactions does not seem 'manufacturing friendly' and scalable. The objective of this investigation was to identify and assess bioprocessing challenges to surmount before embracing *C. reinhardtii* microalgae as a viable system for protein production.

4.3 Material and methods

4.3.1 Cultivation of *C. reinhardtii* MT51 strain

C. reinhardtii expressing MT51 immunotoxin was grown under heterotrophic (in the dark) or mixotrophic (16/8 h light/dark cycle) conditions in TAP (Tris-acetate-

phosphate) medium at room temperature in a rotary shaker at ~125 rpm. The initial inoculum for liquid cultures was obtained from the biomass grown on agar plates for ~ 2 weeks without light exposure. Agar (Sigma A5054) plates were prepared with TAP media containing 100μg/mL kan. Algal biomass from a single plate was resuspended in 25 mL of TAP media and transferred to 150 mL fresh media after 3 days of growth. Subsequent transfers during scale up were done every ~ 3 days until desired culture volume was reached. The volume of the exponential phase inoculum during scale up was ~ 10%. For cultures growing under mixotrophic conditions, the photon irradiance for cultures volumes between 150 and 500 mL was kept at 50 μmol/m^2s (PPF). Once cultures volume reached 500 mL, the light intensity was increased to 75 μmol/m^2s.

Growth and cell concentration was monitored daily by counting cells using a hemocytometer (BrightLine, Hausser Scientific, Horsham, PA) and by measuring optical density at 750 nm wavelenght.

4.3.2 Cultivation of Pfs25 strain

C. reinhardtii strain producing Pfs25 antigen was first grown for ~ 2 weeks without light exposure in agar plates prepared with TAP media containing 150 μg/mL kan. Liquid cultures were grown in TAP medium at room temperature in a rotary shaker at ~ 125 rpm. The initial inoculum for liquid cultures was obtained from the biomass grown on agar plates after ~ 2 weeks. Algal biomass from a single agar plate was resuspended in 100 mL of TAP media without kan and grown for 3 days. Scale up of cultures was performed using 10 % inoculum in the exponential phase (100 mL) in 1-L of fresh TAP

media containing 25μg/mL kan. One liter cultures were grown heterotrophically (in the dark) for 6 days reaching ~ 3 to $5*10^5$ cells/mL. After 6 days, the biomass from 1-L cultures were resuspended in 1-L of fresh TAP media containing 25μg/mL kan and let it grow for ~ 1 day to reach ~10^6 cells/mL. Cultures at ~10^6 cells/mL were ready to be exposed to light to induce recombinant protein synthesis.

Cell growth and cell concentration was monitored daily by counting cells using a hemocytometer (BrightLine, Hausser Scientific, Horsham, PA) and by measuring optical density at 750 nm wavelenght.

4.3.3 Induced expression of recombinant proteins

Immunotoxin MT51: recombinant immunotoxin production was induced in cultures grown under heterotrophic (in the dark) and mixotrophic (16/8 h light/dark cycle) conditions separately at constant illumination but different light exposure times. Immunotoxin MT51 accumulation was first tested in a culture grown under mixotrophic (16/8 h light/dark cycle) conditions until the end of exponential phase (early stationary phase) where illumination was provided at a photon irradiance of 100 μmol/m^2s for 12 h consecutively.

In addition, accumulation of MT51 was also tested in cultures grown under heterotrophic and mixotrophic conditions simultaneously in separate batches. Two separate 1-L cultures of *C. reinhardtii* MT51 grown heterotrophically (1-L) and mixotrophically (1-L) were split into three equal volumes each (for a total of 6 independent cultures), harvested by centrifugation, and each third of the biomass pellet

was resuspended in 1-L fresh TAP media to ensure sufficient nutrients for protein production during the light exposure period. The first 1-L of resuspended culture (containing one third of the initial biomass) was used as control before light exposure (t=0), while the other two 1-L cultures were illuminated at 100 μmol/m^2s for 6 and 24 h respectively.

Pfs25 antigen: Accumulation of Pfs25 antigen was evaluated in cultures grown under heterotrophic conditions (in the dark) at a cell concentration of ~10^6 cells/mL. Cultures were exposed to light for 12, 24, and 36 hours consecutively at a photon irradiance of 120 μmol/m^2s.

4.3.4 Recombinant protein extraction

C. reinhardtii cultures expressing recombinant proteins were grown in liquid media until they reached a desired cell concentration of ~10^6 cells/mL. At the end of the light exposure period, cells were harvested by centrifugation at 10,000 ×g for 15 min at 4 ºC. Pelleted algal biomass was washed with fresh TAP media, weighted, and then resuspended at either 1:10 or 1:5 biomass to lysis-buffer ratio. MT51 cells were lysed by sonication for 1.5 min with 30 s on/off intervals at 4^{o}C using sonicator (Sonifier 250, BRANSON, USA) at 30% output control and 30% duty cycle with a micro probe (1/8' microtip A3-561 BRANSON, USA). For Pfs25 extraction, sonication time was increased to 8 min with 30 seconds on/off sonication intervals to prevent heat accumulation.

Lysis buffer composition and pH depended on the extraction objective and recombinant protein to be recovered (MT51 or Pfs25). The three lysis buffers that were

used in this study were: 1) 100 mM Na_2HPO_4*12 H_2O buffer containing 300 mM NaCl and10 mM EDTA at pH 7.5, 2) 100 mM CH_3COONa buffer containing 300 mM NaCl, and 10 mM EDTA at pH 4.5, and 3) 50 mM Tris-HCl buffer containing 400 mM NaCl, and 0.5% Tween, pH 8.0. All buffers contained a complete protease inhibitor cocktail (Roche-Mannheim, Germany) dissolved in 50 ml buffer for the first two buffers and in 200 mL for the third buffer. Cell lysates were centrifuged (10,000 x g for 10 min) to produce clarified crude extracts.

4.3.5 Recombinant protein purification

Crude extracts were sterile filtered using Polyethersulfone (PES) 0.45 μm filter and mixed with anti-FLAG affinity resin (Sigma Aldrich A4596) equilibrated in the same lysis buffer used for protein extraction. Approximately, 1 mL of resin was used per every 10 g of wet algal biomass. Binding of the recombinant protein to the affinity resin was performed for 2 h at 4 ºC by continuous end-over-end mixing in a Glas Col rotor at ~ 33 rpm (40 % speed control). Affinity resin was washed with 6 column volumes (CV) of loading buffer (lysis buffer). The washed FLAG resin was transferred into Bio Spin disposable chromatography columns (Bio Rad, Cat # 732-6008) for protein elution. Recombinant protein was eluted at pH 3.5 using 5 CV of 100 mM glycine buffer, pH 3.5 that contained 400 mM NaCl. Eluted protein fractions were collected in 5 tubes containing a predetermined amount of 1M Tris-HCl, pH 8.0 to immediately increase the pH of eluted protein and avoid protein denaturation. Anti-FLAG affinity resin was rinsed with 10 CV of phosphate-buffered saline (PBS) and stored at 4 ºC in 50 % glycerol in

TBS + 0.2 % Tween + 0.2% NaN_3 solution buffer containing 50 % glycerol, 0.2 % NaN_3,and 0.05 % Tween. The entire purification assay was performed in cold. Extraction buffer and all the materials used including the sonication probe (1/8' microtip A3-561 BRANSON, USA) were cooled in advance.

4.3.6 Western blot and SDS-PAGE assays

Detection of recombinant proteins in crude extracts and after purification was performed by using anti-ETA antibodies for the immunotoxins and anti-FLAG for the FLAG tagged MT51 and Pfs25 recombinant proteins. The NuPAGE Novex Bis-Tris pre-cast gradient gels (4 - 12 %) from Invitrogen™ were used for electrophoresis following a procedure recommended by the manufacturer.

Reducing-buffer was prepared using LDS sample buffer (4X) (NuPAGE NP0007) containing 10% of reducing agent (Cat no. NP0004). Reduced samples were prepared using a 1:4 ratio reducing-buffer : sample and heated at 70 ºC for10 min. Protein separation was achieved in precast NuPAGE Novex 4-12% Bis-Tris Mini Gels (1.5 mm x 10 wells), Cat no. NP0335BOX). MES SDS Running Buffer (20X) (Cat no. NP0002) stock solution was used to prepare 1X running buffer in RO water. Antioxidant (NuPAGE NP0005) was added to ensure reduced samples during electrophoresis. Gels were run for 35 min at constant voltage (200 V) and then transferred to nitrocellulose membranes using iBlot® 7-Minute Blotting System, Life Technologies Corporation.

After protein transfer to nitrocellulose membrane, the membrane (free sites) was blocked with 2.5 % non-fat milk in TBS containing 0.05 % Tween 20 at pH 7.5 buffer

for 1 h to prevent nonspecific binding of the detection antibodies. Recombinant immunotoxin (MT51) was detected with anti-ETA primary antibody at a concentration of 1:20,000 followed by secondary antibody (horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody) at a concentration of 1:10,000. The incubation time with the primary antibody was 2 h followed by 1h incubation with the secondary antibody. The membrane was washed with TBS containing 0.05 % Tween 20 at pH 7.5 buffer and developed with, 4-chloro-1-naphtol in methanol solution (4-CN) for visualization.

FLAG-tagged recombinant proteins (MT51 and Pfs25) were detected by using anti-FLAG M2-AP (alkaline phosphatase conjugated) antibody from Sigma Aldrich (cat # A9469) at a concentration of 1:1,000. After incubation with the antibody for 1 h, the membrane was washed with TBS containing 0.05 % Tween 20 at pH 7.5, buffer and blots were visualized (developed) with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) (Sigma FAST B5655) dissolved in 10 mL of filtered RO water.

4.3.7 Total soluble protein determination

Total soluble protein from crude extracts and purified samples were quantified using the microplate protocol (working range from 1 to 25 µg/mL and 25 to 1500 µg/mL) Coomassie plus (Bradford) assay kit (Thermo Scientific). Absorption at 595 nm was measured using the $VERSA_{max}$ microplate reader.

4.3.8 Proteolytic activity determination

Novex10 % gelatin and 12 % casein zymogram gels were used to determine the presence of proteolytic activity in crude extracts containing recombinant protein. Crude extract samples were diluted 2-fold with Novex Tris-Glycine SDS Sample Buffer (2X) cat LC2676, without heating or reducing the samples. Running solution used was 1XTris-Glycine SDS running buffer in deionized water. Gels were developed with 100 mL (1X) solution prepared using zymogram renaturing buffer (10X) and Novex zymogram developing buffer (10X) in deionized water. The pH of the developing solution was ~ 6.0. For maximal sensitivity the gels were incubated at 37 ºC overnight.

4.4 Results and discussion

4.4.1 Total soluble protein determination

Previous studies with various transgenic plant tissues demonstrated that extraction pH can be used to reduce the amount of extracted soluble plant proteins (Wilken and Nikolov, 2012). Majority of plant cell proteins are acidic (pI <7) and protein extraction with acidic buffers typically reduces soluble protein in the extract at least two fold. We first compared total protein extraction of *C. reinhardtii* MT51 strain at pH 4.5 and 7.5 (Figure 4.3), and confirmed that low pH extracts contained almost 4-fold less soluble protein that pH 7.5 ones. Thus, assuming recombinant protein is efficiently extracted and stable at pH 4.5, the total protein purification burden in the subsequent downstream processing steps (filtration and chromatography) could be significantly reduced i.e., the amount of recombinant protein expressed as % extracted TSP would be four times

greater at pH 4.5 than pH 7.5. As expected, algal biomass exposed to additional 12 h of light at the end of the mixotrophic period produced additional 50-80 % soluble protein (Figure 4.3).

Cell lysates obtained by sonication for 30 s and 1.5 min did not contain different amounts of TSP at either pH indicating that soluble protein release could be completed in 30 s. To avoid potential batch-to-batch extract variations, cell disruption of MT51 strain was performed for 1.5 min with 30s on/off sonication intervals.

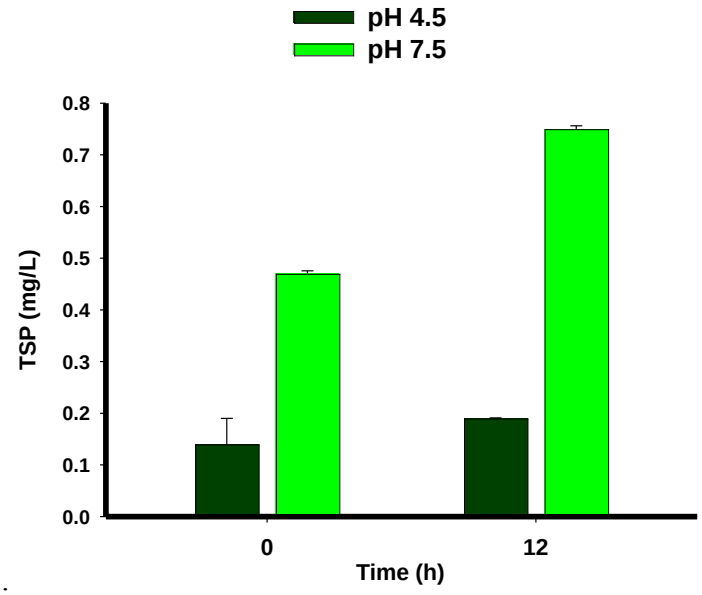


Figure 4.3. Total soluble protein extracted from *C. reinhardtii* MT51 strain.

4.4.2 Extraction and detection of MT51 protein

Detection of MT51 immunotoxin in crude extracts at pH 4.5 and 7.5 was first carried out using anti-ETA antibody (1:20,000), which targets the toxin part of the

immunotoxin. No immunotoxin MT51 bands were seen on the blot from the extract samples at pH 4.5 and pH 7.5 suggesting that MT51 concentration in the extracts was very low (Figure 4.4, lanes 7 to 10). Based on purified MT51 immunotoxin (10 to 500 ng), which was loaded and detected on the same blot (Figure 4.4, lanes 2 to 6), we concluded that the amount of immunotoxin present in the crude extracts was less than 50 ng.

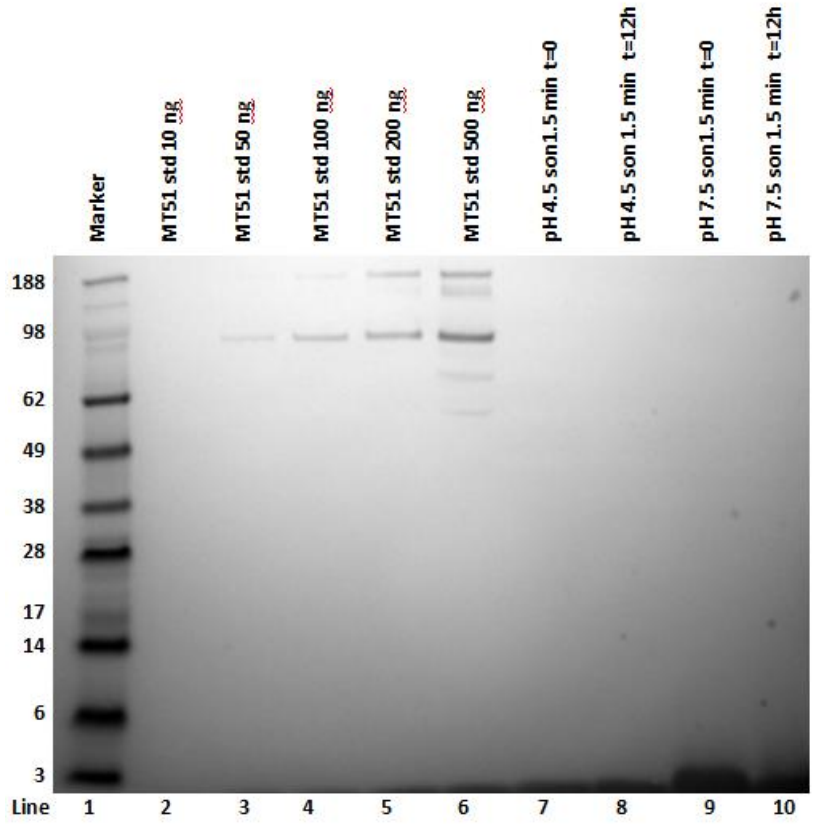


Figure 4.4. Anti-ETA detection of MT51 immunotoxin by Western blot. Lane 1: molecular weight marker, lanes 2-6: are different amounts of purified MT51 immunotoxin, lane 6: MT51 in the extract at pH 4.5 after 1.5 min sonication before light exposure (t=0) used as control, lane 7: MT51 in the extract at pH 4.5 and 1.5 min sonication time after 12 h light exposure, lane 8: MT51 in the extract at pH 7.5 after 1.5 min sonication time before light exposure (t=0) used as control, and lane 9: MT51in the extract at pH 7.5 and 1.5 min sonication time after 12 h light exposure at 100 µmol/m^2s.

The second method to detect recombinant immunotoxin MT51 in the crude extracts utilized anti-FLAG-AP antibody which targets the affinity FLAG-tag attached to the N-terminus of the molecule. The detection of immunotoxin MT51 was done using the same extract samples prepared for previous experiments with anti-ETA. Results shown on Figure 4.5 suggest that anti-FLAG-AP conjugated antibody would work well for detection of FLAG-tagged proteins and it was adapted as a convenient detection method for this study. Western blot in Figure 4.5 shows a somewhat different pattern of extracted and degraded MT51. The difference between extracted immunotoxin at pH 4.5 and 7.5 is best seen by comparing bands in lanes 6 and 7 for pH 4.5 and lanes 8 and 9 for pH 7.5 extracts. The extracts at pH 4.5 did not have detectable MT51 protein bands corresponding to ~ 98 kDa monomer and/or immunotoxin dimer (~ 190 kDa). The only visible FLAG-tagged bands detected in pH 4.5 extracts were ~30 kDa N-terminal fragment (anti-CD22scFv) and unidentified 14 kDa protein, which cross reacted with FLAG antibodies. Extracts at pH 7.5, in addition to the 30 and 14 kDa fragments, had barely detectable monomer and stronger immunotoxin dimer bands. By comparing immunotoxin fragments present in extracts at pH 4.5 and 7.5 to purified MT51 in lanes 2-5, one can conclude that the full length immunotoxin was degraded to proteolytically more stable 30 and 14 kDa fragments. The presence of an unreduced 190 kDa dime band at pH 7.5, but not at pH 4.5, may indicate that *in vitro* proteolysis at pH 7.5 was significantly reduced due to suboptimal pH for protease activity and/or higher protein concentrations in pH 7.5 extracts (Figure 4.3).

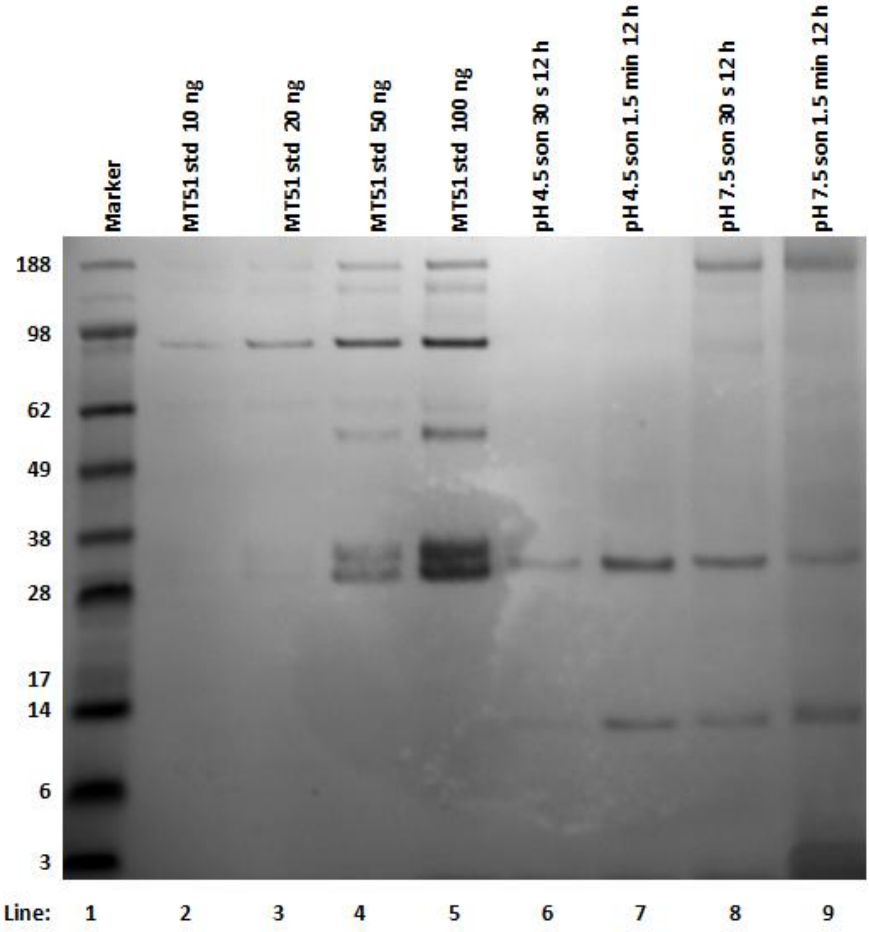


Figure 4.5. Western blot assay of *C. reinhardtii* MT51 using anti-FLAG-AP conjugated antibody. Lane 1: molecular weight marker, lanes 2 to 5: different concentrations of purified MT51 immunotoxin, lane 6: crude extract at pH 4.5 and 30 s sonication time, lane 7: crude extract at pH 4.5 and 1.5 min sonication time, lane 8: crude extract at pH 7.5 and 30 s sonication time, and lane 9: crude extract at pH 7.5 and 1.5 min sonication time. Light exposure time for all the samples in lanes 6 to 9 was 12 h at a photon irradiance of 100 μmol/m^2s.

The existence of proteolytic activity in crude extracts and affinity purified immunotoxin (MT51) samples was tested by running non-denaturing gelatin and casein zymogram gels. A greater proteolytic activity (larger white bands) was observed in extracts at pH 4.5 than pH 7.5 (Figure 4.6). And, it appears that gelatin (Figure 4.6a,

lanes 4 and 5) was a better substrate for detection of proteases in the extracts than casein (Figure 4.6b, lanes 4 and 5). Purified MT51 sample also contained some proteolytic activity – a thin white band on the upper half of the gelatin gel (Figure 4.6a, lane 1). To protect the purified immunotoxin from further degradation during storage, BSA (0.1 %) was add to the MT51 sample.

Although it is difficult to conclude how much of detected degradation products were generated *in vivo*, it appears that further degradation could occur if extractions were conducted at pH 4.5. If acidic proteases in crude extracts were the cause for MT51degradation, one way to control their activity is to perform extractions at pH 7.5 or 8.0, and quickly capture the immunotoxin protein by a different resin than the slow-binding anti-FLAG resin. A better alternative is to develop protease deficient strains, which is one of the project tasks for our collaborator (S. Mayfield).

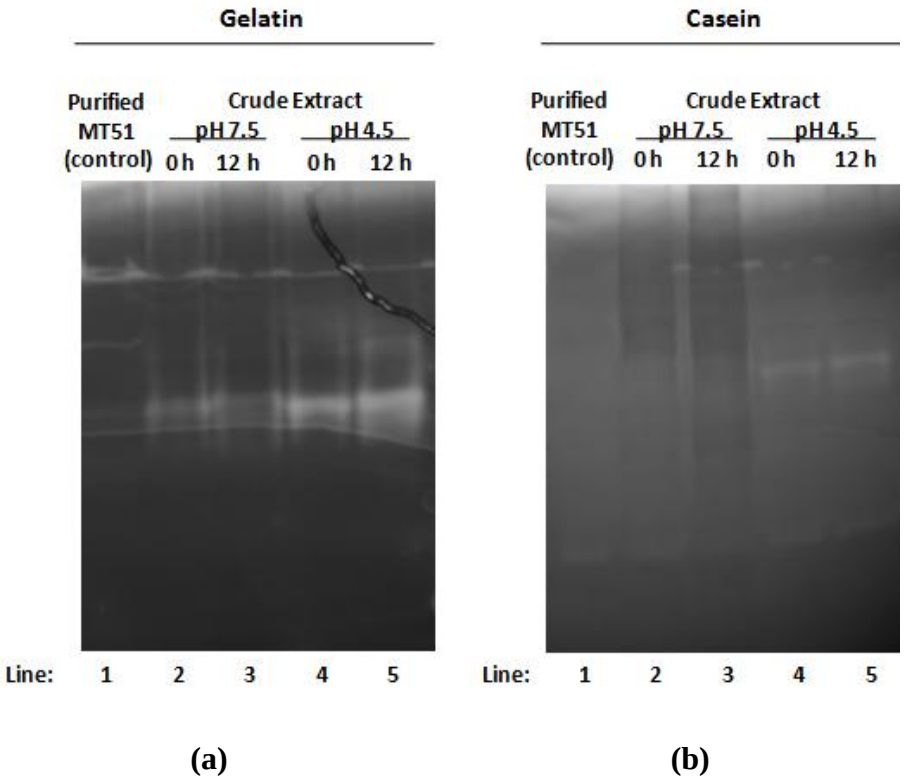


Figure 4.6. Gelatin and casein zymogram gels showing proteolytic activity in purified MT51 sample and clarified extracts at pH 4.5 and 7.5. (a) gelatin zymogram and (b) casein zymogram.

4.4.3 Optimization of growth conditions for maximal accumulation of MT51 immunotoxin in *C. reinhardtii* chloroplast

Since transgene expression is regulated by light-inducible *psbA* promoter, we decided to investigate the effect of biomass growth followed by light-induced MT51 synthesis on MT51 stability and accumulation. For this reason, mixotrophic and heterotrophic growth of *C. reinhardtii* MT51 strain were compared. The purpose of these experiments were:1) to determine if biomass and total protein accumulation during heterotrophic and mixotrophic *C. reinhardtii* growth were different, 2) to confirm that

immunotoxin (MT51) is not produced under heterotrophic growth conditions before light exposure, and 3) to determine if illumination of heterotrophically grown biomass would result in more MT51 accumulation (less degradation) than mixotrophically grown biomass.

Unfortunately, the results of these experiments summarized in Table 4.1 and Figure 4.7 did not provide all the sought after answers. Mixotrophic and heterotrophic cells growth were monitored daily by measuring optical density and cell count before and after light exposure (Table 4.1). Optical density at 750 nm and TSP in heterotrophic and mixotrophic cultures increased as the time of light exposure increased, which agrees with the fact that Rubisco and other light-activated enzymes and pigments are being synthesized under light conditions (Boyle and Morgan, 2009). Cell concentration (# cells/mL) remained almost unchanged during the light period, which might be explained by the higher energetic demand for carbon fixation, since carbon uptake flux is been directed through the Calvin cycle. Boyle and Morgan (2009) estimated *C. reinhardtii* biomass yield under heterotrophic conditions to be 15 g per mol carbon. Under autotrophic conditions biomass yield was 30 g per mol carbon - the maximum yield that can be obtained based on the elemental analysis of *C. reinhardtii*. Therefore, about 50 % of the carbon taken by the cell is used for energy synthesis rather than biomass production; likewise, during mixotrophic growth cells use acetate to grow and some of the carbon is used for energy production rather than biomass production; during mixotrophic growth biomass yield is ~ 23 g per mol carbon (Boyle and Morgan, 2009). These numbers may explain our observation that there was no biomass growth during

the light exposure time due to a higher energy demand for carbon fixation during the Calvin cycle (Boyle and Morgan, 2009; Griffiths et al., 2011).

Table 4.1. Biomass and total protein accumulation in *C. reinhardtii* MT51 strain grown under heterotrophic and mixotrophic conditions

	Heterotrophic growth				Mixotrophic growth			
Sample	**O.D 750 nm**	**Cell conc. (cell/mL)**	**Biomass (g)**	**TSP (mg/mL)**	**O.D 750 nm**	**Cell conc. (cell/mL)**	**Biomass (g)**	**TSP (mg/mL)**
MT51 before light exposure (t=0)	0.19	4.2×10^5	1.0	4.5 ± 6.0	0.17	2.1×10^5	1.3	4.9 ± 1.3
MT51 after 6 h light exposure	0.21	5.2×10^5	1.0	5.5 ± 16.4	0.18	2.1×10^5	1.3	5.8 ± 6.0
MT51 after 24 h light exposure	0.35	6.0×10^5	1.5	7.9 ± 15.8	0.42	1.8×10^5	1.7	8.3 ± 5.5

Results presented in Figure 4.7 essentially show almost complete degradation of synthesized MT51. The absence of protein bands around 98 or 190 kDa (dimer form) suggests no immunotoxin synthesis under heterotrophic growth before light exposure (Figure 4.7, lane 2). Also, heterotrophic cell extracts did not contain any of MT51 derived fragments except for the 14 kDa protein. Based on the 30 kDa degradation product one can conclude that during the heterotrophic growth (t=0, control) there was no significant MT51 synthesis. The first rather faint 30 kDa band appeared after 6 h exposure (Figure 4.7, lane 4) and a stronger one after 24 h (Figure 4.7, lane 6). The culture grown under mixotrophic conditions (Figure 4.7, lane 3) shows a barely detectable protein band at ~ 190 kDa even before light exposure confirming that the *psbA* promoter was turned on during the intermittent light exposure. Mixotrophic culture extracts also contained a 30 kDa band at t = 0 h, which increased slightly in density after exposing the culture to light for 6 (Figure 4.7, lane 5) and 24 h (Figure 4.7, lane 7). Storage of the crude extracts at 4 ºC for a week resulted in additional degradation of higher MW bands as judged from the greater density of 14 kDa bands (Figure 4.7, lanes 8-10).

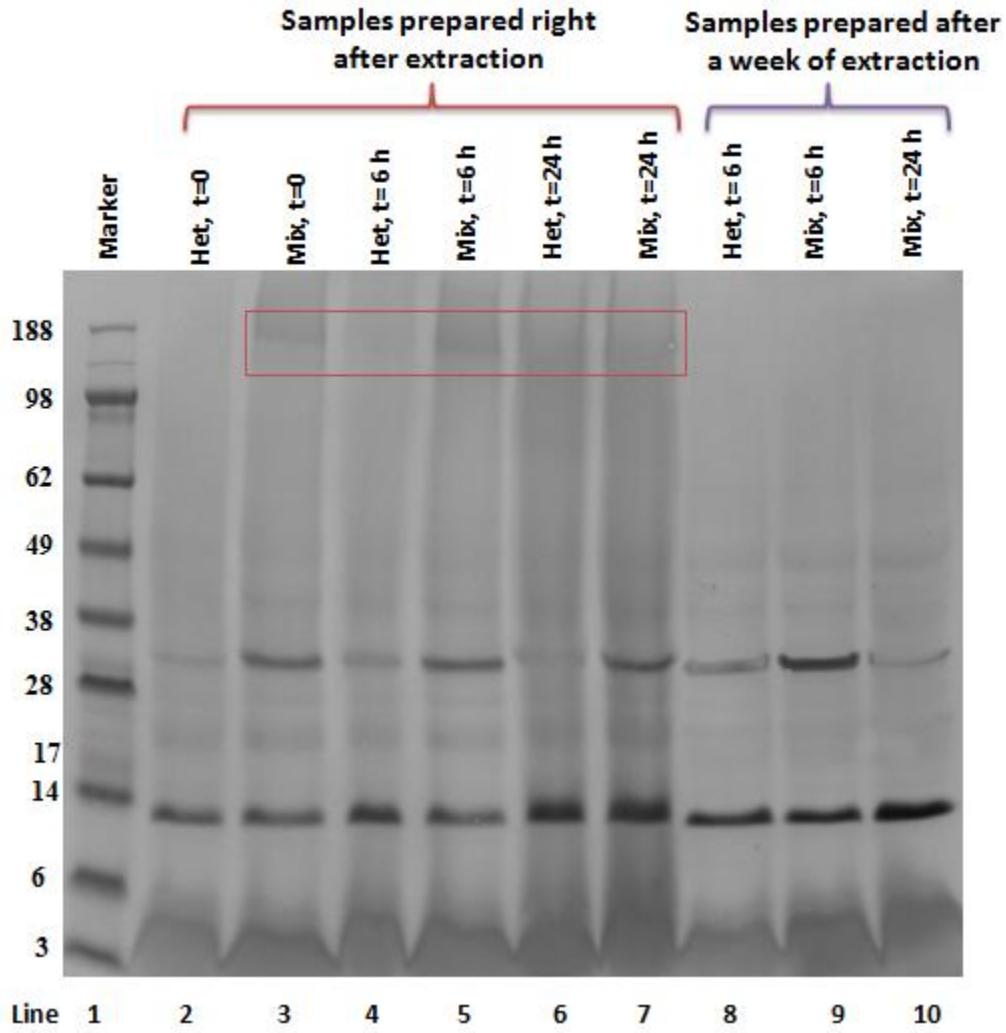


Figure 4.7. Western blot of crude extracts from *C. reinhardtii* algal biomass grown under mixotrophic and heterotrophic conditions. Immunotoxin MT51 production was induced by light exposure for 6 and 24 h and detected using anti-FLAG-AP conjugated antibody. Lane 1: molecular weight marker; lanes 2 and 3: MT51 in the crude extract before light exposure (t=0) under heterotrophic and mixotrophic growth conditions respectively; lanes 4 and 5: MT51 in the crude extract after 6 h of light exposure under heterotrophic and mixotrophic growth conditions respectively; and lanes 6 and 7: MT51in the crude extract after 24 h of light exposure under heterotrophic and mixotrophic growth conditions respectively. Samples ran in lanes 2 to 7 were prepared right after extraction process while samples run in lanes 8 to 10 were prepared from the same extracts after one week storage at 4 ºC. Algal biomass was sonicated for 1.5 min and photon irradiance was 100 µmol/m^2s.

In summary, the significant *in vivo* and *in vitro* degradation of MT51 immunotoxin clearly requires the development of protease deficient strains before undertaking any purification process development. For these reasons, we have decided to focus algal bioprocess development using *C. reinhardtii* Pfs25 strain, which was developed using similar transgene construct and vector as the MT51 strain for the expression of *Plasmodium falcaparum* surface protein 25. Gregory et al., (2012) have established that Pfs25 is more stable in harvested transgenic tissue and crude extracts than MT51, which should allow collecting relevant bioprocessing data for the development of *C. reinhardtii* chloroplast expression system.

4.4.4 Extraction and purification of recombinant Pfs25 protein

Based on extraction and stability data obtained with MT51, we selected to use extraction buffer at pH 8.0 containing 50 mM Tris-HCl, 400 mM NaCl and 0.5 % Tween 20. Preliminary screening of extraction and purification conditions of Pfs25 antigen was performed using two 20-g batches of frozen (-80 ºC) biomass obtained from our collaborator (Dr. Mayfield, UCSD). Protein extraction was performed at different biomass to lysis buffer (1:10 and 1:5) ratio to determine the effect of crude lysate concentration on recovery yield and purity of the bound and eluted Pfs25 from FLAG affinity resin. Figure 4.8 summarizes extraction and purification of Pfs25 performed at 1:10 and Figures 4.9 and 4.10 at 1:5 biomass-to-buffer ratio. From the Western blot in Figure 4.8b, lane 3, it appears that some loss of Pfs25 has occurred during resin wash before protein elution. Most of the recombinant protein was eluted in three fractions

(fractions 2, 3, and 4) as indicated by the SDS-PAGE gel (Figure 4.8a, lanes 4 to 6) and confirmed by Western blotting (Figure 4.8b, lanes 5 to 7). The results from the 1:5 extraction were similar (Figures 4.9 and 4.10), although the initial concentration of Pfs25 in the lysate seemed lower in the 1:5 extract (Figure 4.9b, lane 2) than 1:10 (Figure 4.8b, lane 2). Majority of Pfs25 protein was eluted in two column volumes (fractions 2 and 3) as shown by SDS-PAGE (Figure 4.10a, lanes 3 and 4) and confirmed by western blot (Figure 4.10b, lanes 3 and 4).

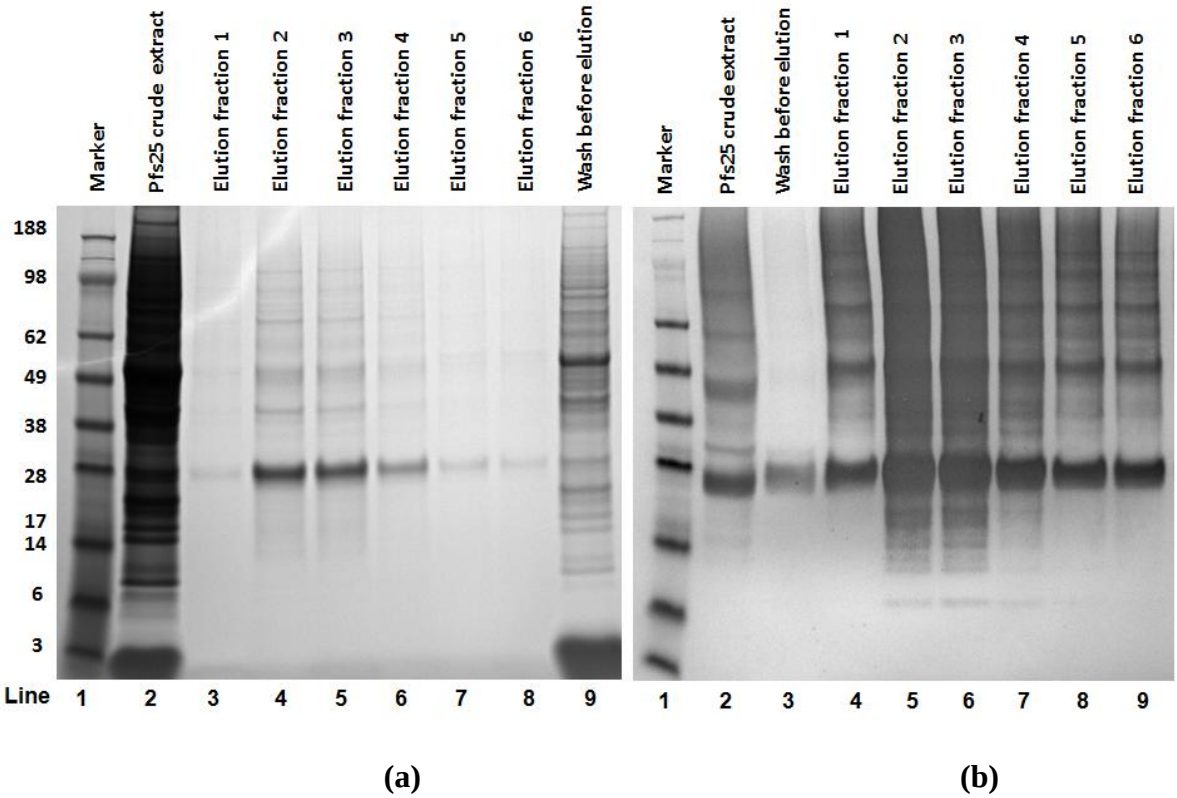


Figure 4.8. Extraction and purification of Pfs25 antigen with anti-FLAG affinity resin using 1:10 biomass:lysis-buffer ratio. a) SDS-PAGE of purified elution fractions and b) Western blo of purified elution samples.

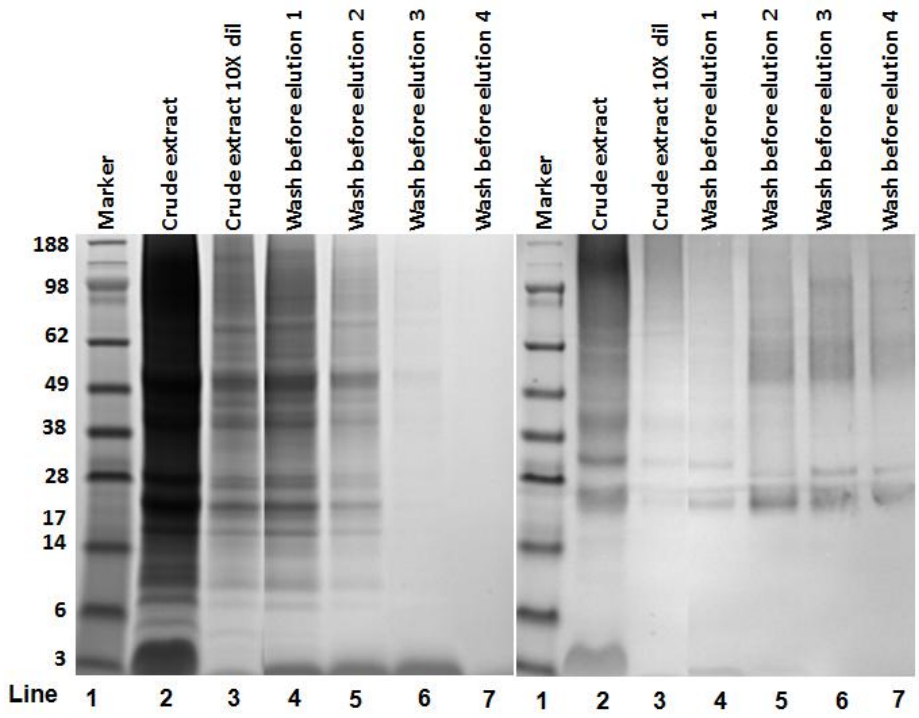


Figure 4.9. Samples from extraction and purification of Pfs25 antigen with anti-FLAG affinity resin using 1:5 biomass:lysis-buffer ratio. a) SDS-PAGE of crude extract and washes before elution step and b) western blot of crude extract and washes before elution step.

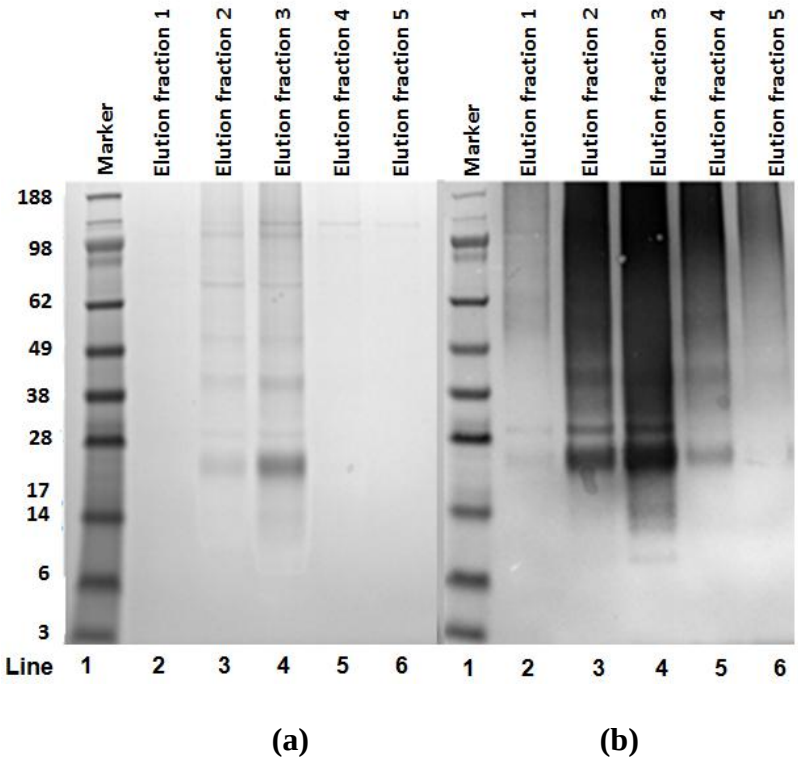


Figure 4.10. Purification of Pfs25 antigen with anti-FLAG affinity resin using 1:5 biomass:lysis-buffer ratio. a) SDS-PAGE of purified fractions and b) western blot of purified fractions.

Protein concentration of eluted fractions from the affinity column are plotted in Figure 4.11 and summarized in Table 4.2. The amount of Pfs25 monomer (25 kDa band) in the eluted fractions was estimated from SDS-PAGE gels using ImageJ software (Rasband, 1997-2012) and reported in Table 4.2 as Pfs25 purity. The estimated average purity of Pfs25 in the eluted protein from 1:10 extracted batch was 57 % and 72 % from the 1:5 batch. Based on SDS-PAGE and Western blots, protein impurities consisted of Pfs aggregates (~ 50 and 75 kDa) and other unidentified proteins all of which were better visible in the Figure 4.8 than Figure 4.10. There was almost 2-fold more protein

recovered from 1:10 than 1:5 extraction (Table 4.2), confirming our previous comment that the two frozen algal biomass batches had seemingly different Pfs25 accumulation levels. This somewhat unexpected outcome underlines the importance of achieving a solid understanding and control of biomass growth and induced expression conditions to minimize batch-to batch variations - a critical prerequisite to any downstream process development venture.

Table 4.2. Protein concentration (TSP) of eluted Pfs25 fractions

Sample	Extraction ratio (1:10)		Extraction ratio (1:5)	
	TSP (µg/mL)	**Vol (mL)**	**TSP (µg/mL)**	**Vol (mL)**
Elution fraction 1	30.2	1	0.0	1
Elution fraction 2	240.5	1	155.0	1
Elution fraction 3	100.1	1	70.20	1
Elution fraction 4	36.9	1	0.0	1
Elution fraction 5	26.5	1	0.0	1
Elution fraction 6	12.4	1	0.0	1
Total protein recovered (µg)	446		225	
Pfs-25 in eluted protein	268		162	
Pfs-25 purity (%)	57		72	

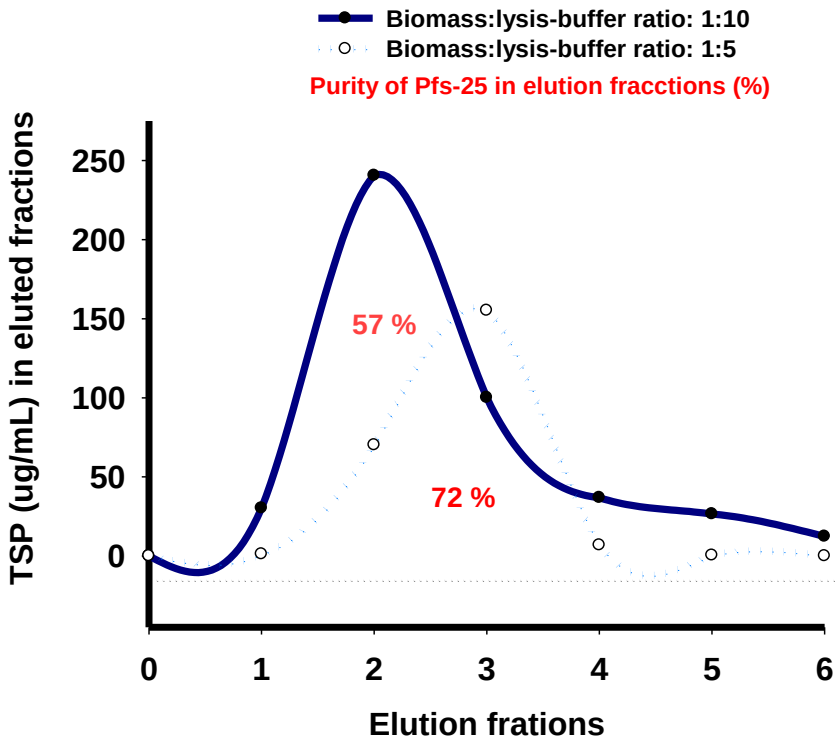


Figure 4.11. Elution profile of proteins bound to anti-FLAG affinity resin. The number under the peak represents an estimated average Pfs25 purity (28 kDa band) in the pool.

4.4.5 Optimization of biomass accumulation in liquid media before light exposure for recombinant protein synthesis

As mentioned before, *C. reinhardtii* Pfs25 strain generated by replacing endogenous *psbA* with the transgene construct (Pfs25), allows optimization of biomass accumulation separately from recombinant protein synthesis. Four transgenic *C. reinhardtii* Pfs25 transformants were screened to select the best Pfs25 producer.

Independent transformants were streaked in TAP media agar plates containing 150 μg/mL kan and grown for ~7 to 10 days. Liquid cultures were started from the cell biomass in agar plates, which was suspended in 100 mL of fresh TAP media (without kan). The liquid culture suspension was incubated in the dark at room temperature and 125 rpm shaking. Of the four transformants tested, only those labeled as pJAG-50 and pJAG-34 grew well in liquid media and were scaled up. The 100 mL cultures of transformants pJAG-50 and pJAG- 34 were incubated in the dark for 3 days reaching concentrations of about $3x10^5$ cells/mL. These cultures were used as inoculum for 1-L cultures, which were then grown for additional 6 days reaching 5×10^5 cells/mL. The cells from both 1-L cell cultures were harvested by centrifugation at 6000 rpm for 6 min, resuspended in 1-L of fresh TAP media containing 25 μg/mL kan and then incubated again for 1 to 2 days under the same conditions (no light, room temperature, and 125 rpm) until they reached ~ 10^6 cells/mL. These cultures, still in the exponential phase, were used as a starting culture material for induced Pfs25 production experiments.

Transformant pJAG-50 grew slower than pJAG-34 in the first 3 days after inoculation of the first 1-L culture. At the 6^{th} day, both reached a similar cell concentration. After 24 h from the last transfer (day 8 of incubation), pJAG-34 had on average slightly greater cell concentration 10^6 compared to 7.5 10^5; however, the cell count of transformant pJAG-50 was somehow difficult due to the formation of 'palmelloid' cells, which are clumps of non-motile cells as a result of a failure in complete separation from the mother cell wall after mitosis (Harris, 2009); therefore, these clusters of cells challenge accuracy of cell count.

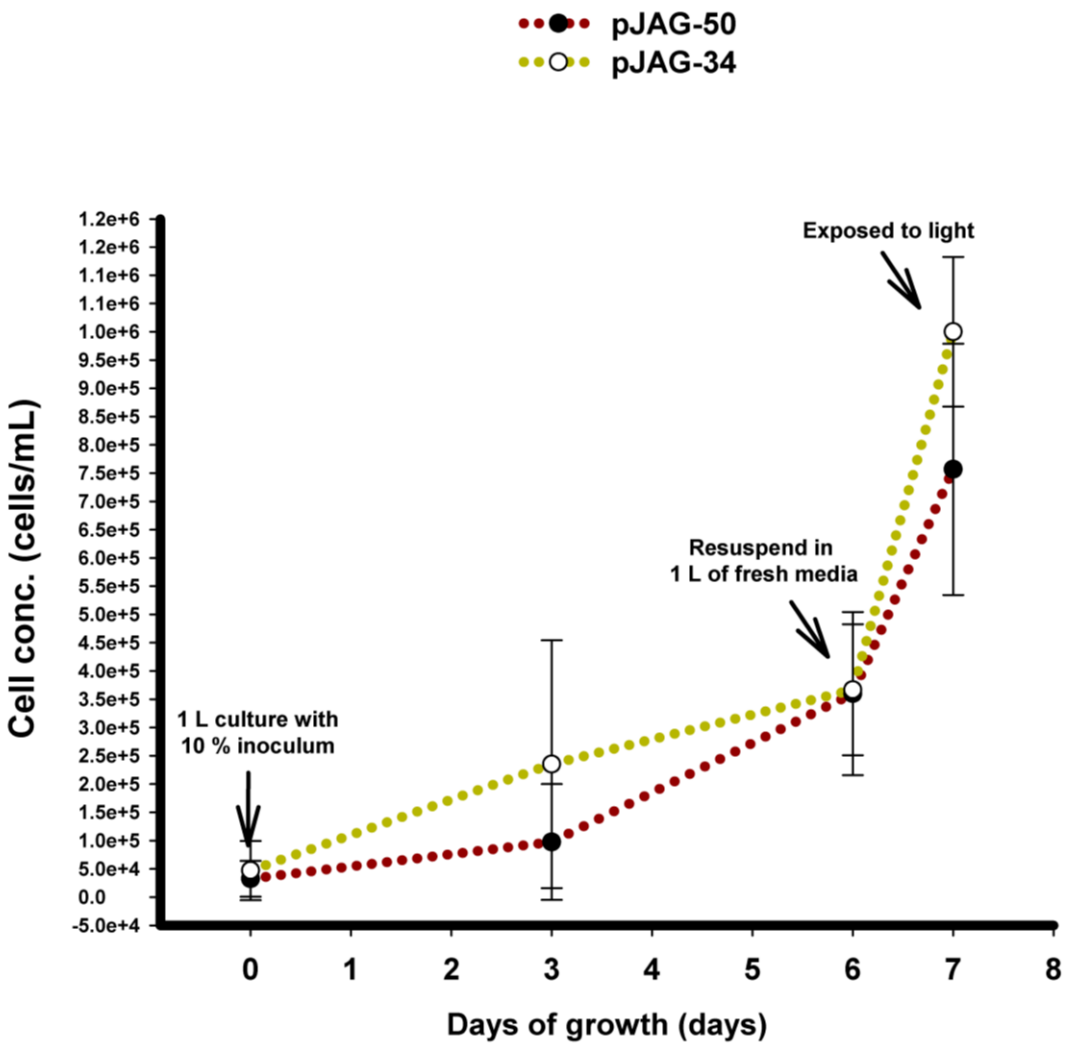


Figure 4.12. Optimization of heterotrophic biomass accumulation of *C. reinhardtii* Pfs25 transformants pJAG-50 and pJAG-34

4.4.6 Induced recombinant Pfs25 accumulation in the chloroplast of *C. reinhardtii*

Because Pfs25 antigen production is regulated by the light inducible *psbA* promoter, the heterotrophic cultures of transformants pJAG-50 and pJAG-34 were exposed to photon irradiance of ~120 µmol/m^2s for 12, 24, and 36 h.

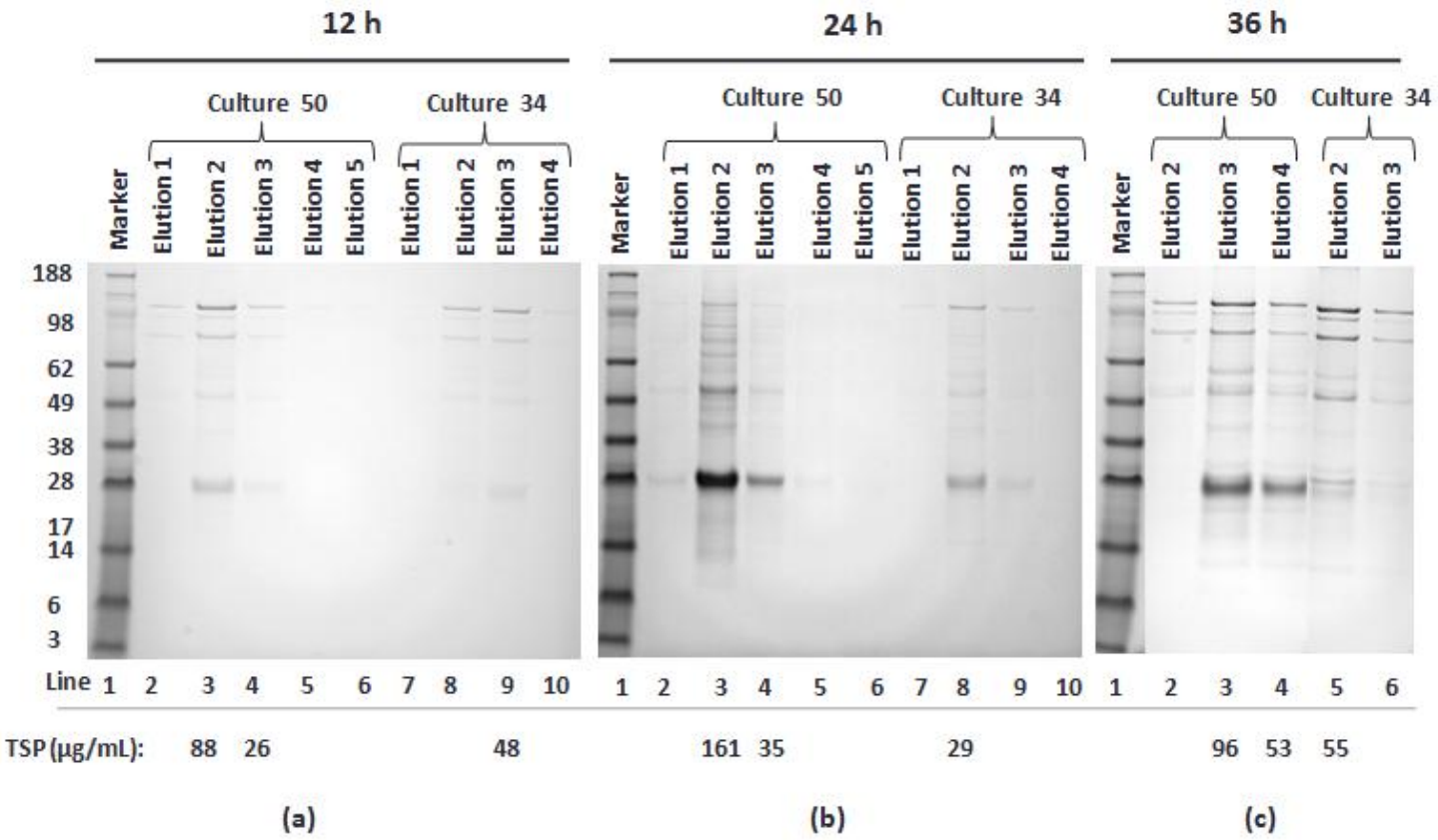


Figure 4.13. SDS-PAGEs of purified fractions from crude extract of *C. reinhardtii* expressing recombinant Pfs25 in the chloroplast after different times of light exposure. Purified protein fractions after (a)12 h light exposure, (b) 24 h light exposure, and (c) 36 h light exposure. Photon irradiance was fixed at 120 µmol/m^2s. Total soluble protein (TSP) of selected elution fractions is shown below the gel.

Recombinant protein produced after different light exposure times was quantified by anti-FLAG affinity purification of FLAG-tagged Pfs25 protein as described in the Materials and Methods section. Purified samples were analyzed by SDS-PAGE, Western blot, and total eluted protein determined by Bradford assay (Bradford, 1976). Protein fractions eluted from the FLAG affinity column after exposure of cultures pJAG-50 and pJAG-34 to light for 12, 24, and 36 h are compared in Figure 4.13. SDS-PAGE and TSP data in Figure 4.13 clearly indicate that transformant pJAG-50 produces more Pfs25 protein (28 kDa band) at all three exposure times than transformant pJAG-34. The highest accumulation of recombinant Pfs25 protein, judged from the 28 kDa band density and TSP in Figure 4.13 b, lanes 3 and 4, was achieved after 24 h of light exposure. Western blotting of the same samples was performed to confirm the identity of Pfs25 (28 kDa band) and to monitor Pfs25 aggregation and/or degradation as a function of the induction time (Figure 4.14). After 36 h of light exposure, the presence of high molecular weight protein bands (~50, 75, and 100 kDa) was detected by SDS-PAGE (Figure 4.13c) as well as by Western blot analysis (Figure 4.14c).

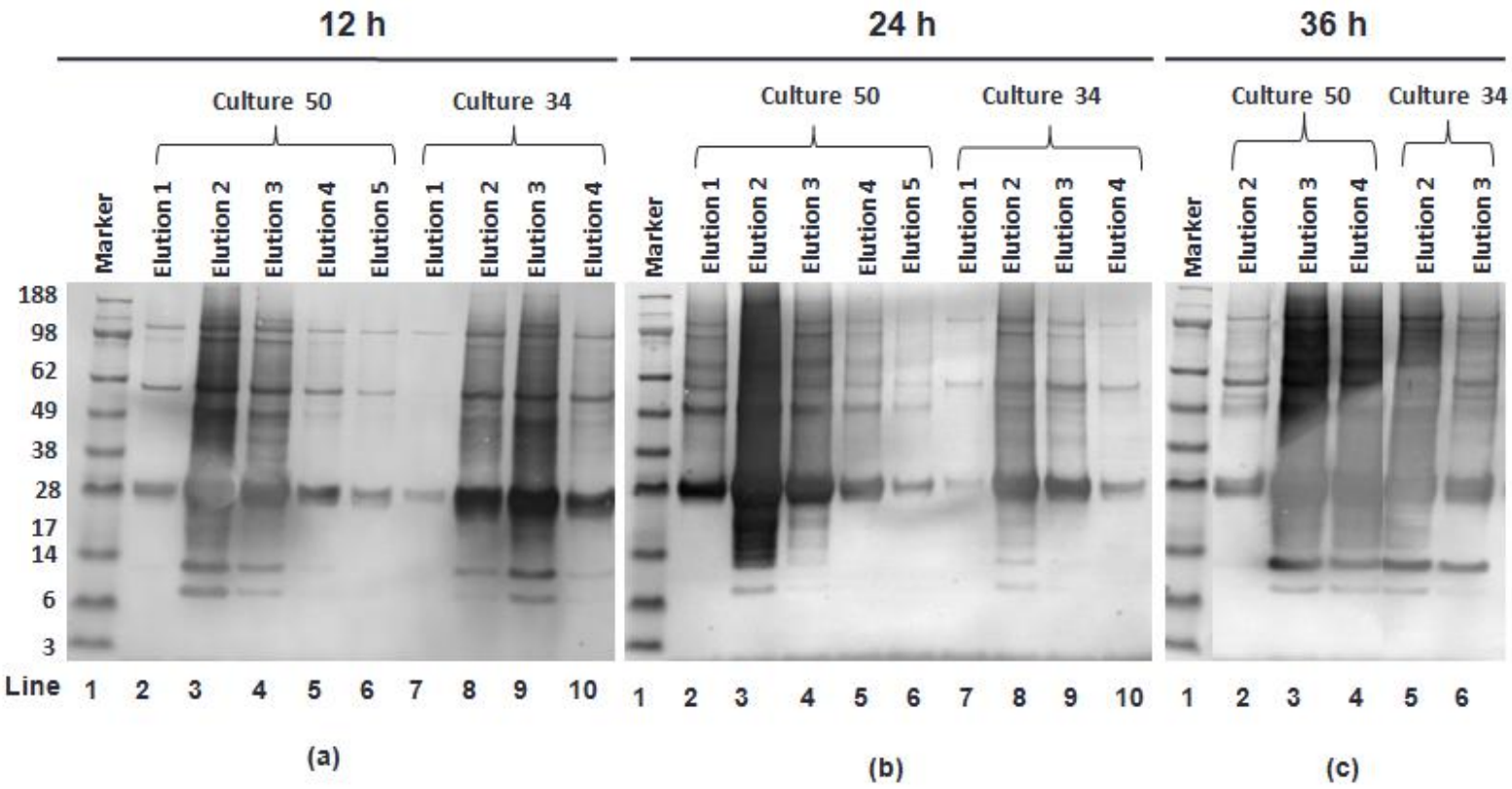


Figure 4.14. Western blots of purified fractions from crude extract of *C. reinhardtii* expressing recombinant Pfs25 in the chloroplast after different times of light exposure. Purified protein fractions after (a)12 h light exposure, (b) 24 h light exposure, and (c) 36 h light exposure. Photon irradiance was fixed at120 µmol/m^2s.

Because MW of these protein impurities were multiples of Pfs25 MW and had N-terminal FLAG-tag, one can conclude that these originated from aggregation of Pfs25. Furthermore, low molecular weight proteins of ~7 and 12 kDa were stronger (more visible) in both cultures (pJAG-50 and pJAG-34) after 36 h than after 24 h light exposure. The appearance of low MW protein fragments suggests proteolytic degradation (Figure 4.14c, lanes 3 to 6).

Purity of recombinant Pfs25 antigen recovered in the elution fractions of anti-FLAG affinity purification was assessed and quantified using ImageJ software. A comparison of total protein recovered in the pool of purified fractions and Pfs25 purity (%) in each pool as function of the induction time is summarized in Figure 4.15. The protein pool of transformant pJAG-50 had the highest protein concentration and highest purity (69 %) in the 24 h pool (Figure 4.15). Light exposure of both cultures for 24 h resulted in consistently better protein production level than at 12 and 36 h, and recombinant protein expression level of transformant pJAG-34 always significantly lower than pJAG-50.

4.5 Summary

Chlamydomonas reinhardtii chloroplast has a demonstrated potential for producing complex recombinant proteins. Several challenges were identified during this study that have to be overcome to make this platform commercially attractive i.e., i) low transgene expression level, ii) proteolytic instability (degradation) of immunotoxin MT51 molecule, and iii) aggregation of Pfs25 antigen.

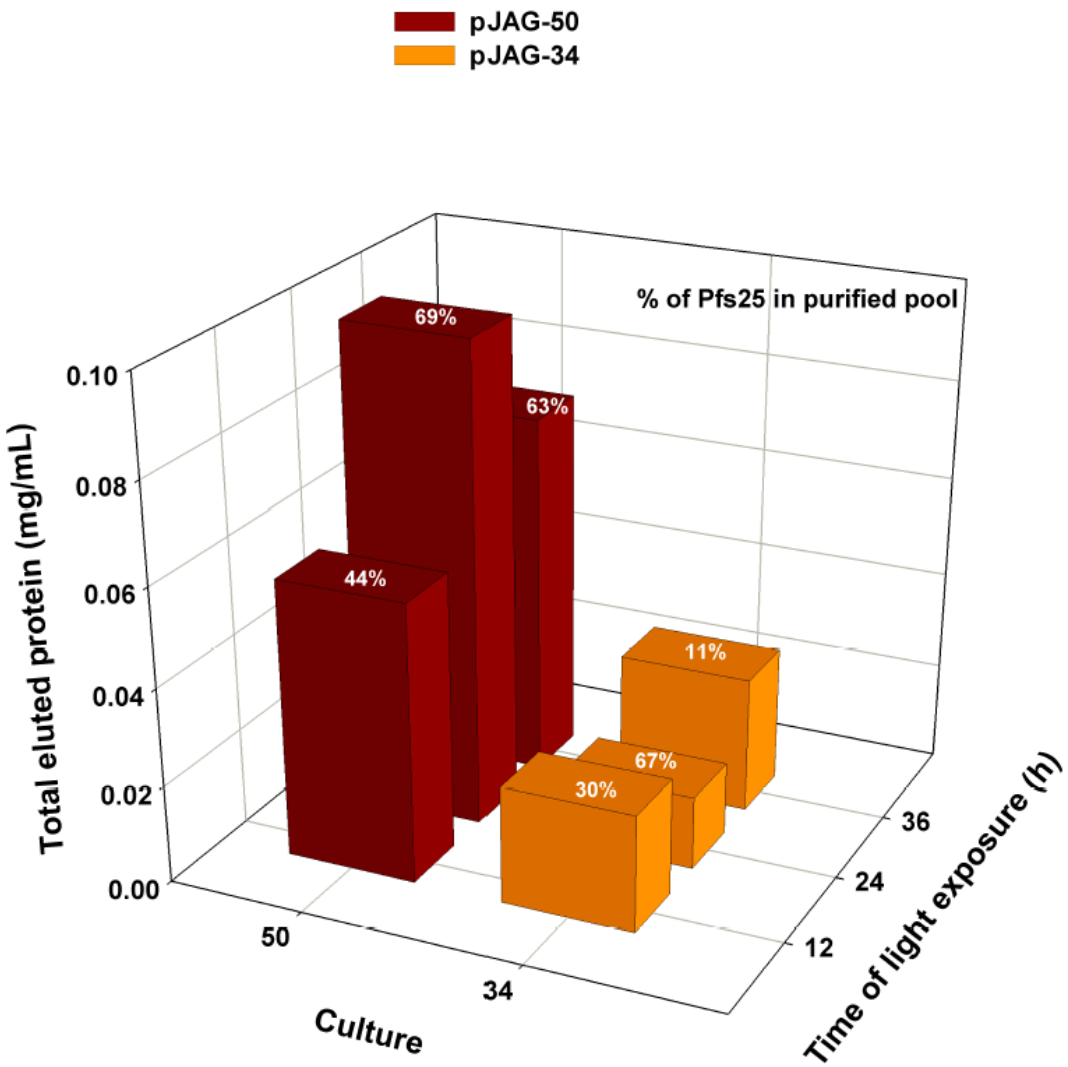


Figure 4.15. Concentration of total protein eluted from anti-FLAG affinity column as function of light exposure time (12, 24, and 36h) and different transformants (pJAG-50 and pJAG-34). Pfs25 protein purity in the pool of purified samples was calculated using ImageJ software and reported as percentage of total band area on SDS-PAGE gels.

Design of a protease deficient *C. reinhardtii* strains to increase stability and accumulation of recombinant proteins is critical. For downstream process development of MT51 product, a 10-fold increase in accumulation level and significantly improved *in vivo* and *in vitro* protein stability would be required. Pfs25 vaccine antigen is relatively stable, but monomer aggregation has to be minimized or controlled during induction period. Some of the approaches used for optimization of recombinant protein production in *E. coli* should be considered and possibly adapted. These might include lowering culture temperature during induction period as well as cloning protein disulfide isomerizes and chaperons.

Accumulation of Pfs25 in the crude extract at 0.1 mg/mL is in the range amenable to process development. The best conditions for optimal accumulation of Pfs25 were: 1) cell cultures in the late exponential phase at 10^6 cells/ml, 2) induction time (light exposure time) of 24 h, and 3) photon irradiance of 120 μmol/m^2s. Anti-FLAG affinity resin used for recovery and purification of both recombinant proteins is suitable for analytical applications but not sufficiently robust for column scale up. Therefore, other affinity tags such His7 and MAT tags should be considered with either recombinant protein to enable robust process purification scale up.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Process development of harvesting and pre-concentration of marine microalgae (*Nannochloris oculata* and *Nannochloropsis salina*) via flocculation requires understanding of chemical interaction between flocculating agent and algae cells, the mechanism of cell aggregate formation, as well as systems properties and variables affecting sedimentation and biomass removal efficiency.

A comprehensive investigation of culture properties and flocculation process variable revealed that AOM, cell concentration, flocculant dosage, ionic strength, media pH, and flocculant (polymer) charge density had an effect on harvesting efficiency but to a different extent. Presence of excreted organic matter in algal cultures had the highest impact on flocculant dosage and harvesting efficiency, independently of the type of flocculant i.e., inorganic salt and natural or synthetic polymers. For example, the concentration of secreted protein in the growth media of *N. salina* after one and two weeks of growth was rather low (4 to 8 mg/L) to significantly interfere with flocculant-cell interaction and tie up extra amount of flocculant. On the other hand, carbohydrate concentration ranging from 100 to 220 mg/L was considerably high and more likely to be the main component of AOM triggering a greater flocculant dosage demand for efficient algal biomass flocculation.

High ionic strength (~ 40 mS/cm), which characterizes growth media of marine microalgae, did not have a significant effect on flocculation efficiency when using an

inorganic electrolyte ($AlCl_3$). However, *N. salina* flocculation with cationic polymers was indirectly affected by media ionic strength and required the application of high-charge density polymers to overcome the presence of electrolytes.

Cell concentration had a significant effect on harvesting efficiency using inorganic electrolyte flocculation, although the needed $AlCl_3$ amount to achieve greater than 90 % removal efficiency was not proportional to cell concentration. For instance, low density cultures (10^6 cells/mL) required 6-fold greater flocculant dosages than cultures with ~ 10^7 cells/mL. Best flocculation conditions were achieved with cultures grown until early stationary phase reaching ~10^7 cells/mL.

The optimal pH for algae flocculation depended on the physico-chemical properties of flocculating agents. For instance, destabilization of the system to induce flocculation when using $AlCl_3$, was achieved at pH 5.3. Optimal flocculation with chitosan required two-steps pH adjustment: acidification to pH 6.5 before chitosan addition and then a pH increased to 8.0 after chitosan addition to initiate algae-polymer co-precipitation. On the other hand, polyacrylamide based synthetic cationic polymers, did not require pH adjustment.

Acidification of algal cultures could be a significant cost burden and selecting a low-buffering-capacity growth media and/or a different microalgae species can mitigate the cost. For instance, *Nannochloropsis salina* algae strain which grows in a low buffering capacity media (F/2), required 21-fold less acid to achieve optimal pH for flocculation with $AlCl_3$ when compared to the amount needed for *Nannochloris oculata,* which grows in a high buffering capacity media that contains $NaHCO_3$ as a carbon source.

Flocculation mechanism varied depending on the type of flocculant. For instance, when using $AlCl_3$ we hypothesize that the destabilization to induce flocculation is caused by partial charge neutralization followed by sweep flocculation mediated by aluminum hydroxide precipitation. The principal mechanism of polymer flocculation is polymer bridging which typically requires less agent compared to charge neutralization or sweep flocculation with inorganic electrolytes.

Chlamydomonas reinhardtii chloroplast has a demonstrated potential for producing complex recombinant proteins. Several challenges were identified during this study that have to be overcome to make this platform commercially attractive i.e., i) low transgene expression level, ii) proteolytic instability (degradation) of immunotoxin MT51 molecule, and iii) aggregation of Pfs25 antigen. Design of a protease deficient *C. reinhardtii* strains to increase stability and accumulation of recombinant proteins is critical.

For downstream process development of MT51 product, a 10-fold increase in accumulation level and significantly improved *in vivo* and *in vitro* protein stability would be required. Pfs25 vaccine antigen is relatively stable, but monomer aggregation has to be minimized or controlled during induction period. Some of the approaches used for optimization of recombinant protein production in *E. coli* should be considered and possibly adapted. These might include lowering culture temperature during induction period as well as cloning protein disulfide isomerizes and chaperons.

Accumulation of Pfs25 in the crude extract at 0.1 mg/mL is in the range amenable to process development. The best conditions for optimal accumulation of Pfs25 were: 1)

cell cultures in the late exponential phase at 10^6 cells/ml, 2) induction time (light exposure time) of 24 h, and 3) photon irradiance of 120 μmol/m^2s. Anti-FLAG affinity resin used for recovery and purification of both recombinant proteins is suitable for analytical applications but not sufficiently robust for column scale up. Therefore, other affinity tags such His7 and MAT tags should be considered with either recombinant protein to enable robust process purification scale up.

5.1 Recommendations for future work

The suggestions for future work include:

i. Test flocculation of stressed microalgae for oil production. Conduct nitrogen starvation stress of selected microalgae species to induce lipid production and then performed flocculation experiments to study the effect of lipid content on harvesting of algal biomass via flocculation.

ii. Induced expression of Pfs 25. Three main suggestions pertaining to Pfs25 accumulation that would be important to address are: i) to scale up the cultures from 1 L to 10 L scale to identify whether scalability is an issue and induce recombinant protein expression under the best conditions found with this study (24 h light exposure time and 120 μmol/m^2s), ii) to develop purification process to achieve more than 90% purity, and iii) to investigate extract pretreatment conditions to enhance purification efficiency. Specifically, perform ammonium sulfate precipitation right after extraction to remove some of the native proteins, acidic precipitation for Rubisco removal, and flocculation

with synthetic cationic polymers to remove negatively charged molecules and native proteins present in the crude extract.

iii. Regarding the immunotoxin work, stability studies using a smaller molecular weight and more stable construct derived from MT51 such as, MT47 would be useful to determine segments of the molecule prone to degradation. MT57 is the single chain of the antibody attached to the variant of exotoxin A (scFv-ETA).

iv. Additionally, to understand where degradation of MT51 and Pfs25 proteins is occurring and determine whether it can be controlled, *in vitro* and *in vivo* degradation should be investigated. For *in vivo* degradation studies, the effect of different photon irradiance for induction of recombinant protein i.e., 80, 100, and150 $\mu mol/m^2s$ after 12, 24, and 36 h light exposure should be tested to determine light-dependent protease activity. *In vitro* degradation during protein extraction and elution from the resin at low pH should also be determined and mitigated. The effect of protease inhibitor cocktail during extraction of both proteins should be considered. The other important factor to analyze would be the effect acidic pH on purified protein samples as a function of time. Degradation of the recombinant protein would be monitored by the detection of lower molecular weight fragments by Western blot and SDS –PAGE.

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